ABSTRACT

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Infectious laryngotracheitis (ILT) is a highly contagious acute respiratory disease of chickens for which safe and efficacious vaccines are not available currently. In the present study, we have generated three recombinant Newcastle disease viruses (rNDV's) expressing three major envelope glycoproteins gB, gC and gD of ILTV individually. A single oculonasal inoculation of chickens with rNDV's elicited detectable level of systemic antibodies specific to ILTV. Following challenge with virulent strain of ILTV, chickens immunized with the rNDV's displayed partial protection with reduced clinical signs and shorter duration of disease compared to the control group. Our data suggested that NDV vectored ILTV vaccines are useful against ILTV infection, but might require augmentation by a second dose or require modification of ILTV glycoproteins which allow them to incorporate into the mature rNDV virions for better induction of humoral and cell mediated immune responses.

IMPROVED INFECTIOUS LARYNGOTRACHEITIS VIRUS VACCINES USING NEWCASTLE DISEASE VIRUS VECTOR

By

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Dedication

I wholeheartedly dedicate this work to my parents for their love and support and to all the scientific staff at PD-ADMAS, ICAR, Bangalore, for their valuable suggestions and great support to pursue my graduate studies.

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I thank my advisor, Dr. Siba K Samal, for a great opportunity to join his lab and full support, guidance and help throughout my degree. His true passion for science has impressed me. Under his generous guidance, I truly believe that I am fully prepared for the next important step in my life. I also like to thank my committee members, Dr. Xiaoping Zhu and Dr. Jeffrey DeStefano for their valuable time, suggestions and support during my graduate study.

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List of abbreviations

aa	amino acid
bp	base pair
CMI	cell mediated immune respone
cDNA	complementary DNA
CPE	cytopathic effect
CEO	chicken embryo origin
DF1	Douglas Foster 1
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxy ribonucleotide triphospahte
EDTA	ethylenediamine tetraacetate
ELISA	enzyme linked immunosorbent assay
EMEM	essential modified Eagle's medium
FBS	fetal bovine serum
GS	gene start
GE	gene end
HA	hemagglutination assay
HDV	hepatitis delta virus
HI	hemagglutination inhibition
HVT	herpes virus of turkey
h	hour
HCl	hydrochloric acid

IgA	immunoglobulin A
IgG	immunoglobulin G
ILT	infectious laryngotracheitis
ILTV	infectious laryngotracheitis virus
kDa	kilo Daltons
KCl	Potassium chloride
L	large polymerase
M	matrix
mRNA	messenger RNA
MDT	mean death time
MEM	minimum essential medium
MOI	multiplicity of infection
NaCl	sodium chloride
NDV	Newcastle disease virus
nm	nanometer
NP	nucleocapsid protein
nt	nucleotide
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PI	post infection

RBC	red blood cell
RNA	ribonucleic acid
rNDV	recombinant Newcastle disease virus
RNP	ribonucleoprotein
RT-PCR	reverse transcription PCR
SARS-CoV	severe acute respiratory syndrome corona virus.
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPF	specific pathogen free
TCO	tissue culture origin
USDA	United States Department of Agriculture

Chapter 1: General introduction

1.1 Introduction

Infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens, which is caused by infectious laryngotracheitis virus (ILTV), a member of the family herpesviridae. The disease is characterized by signs of respiratory depression, gasping, expectoration of blood, mucus, and high mortality [1]. The disease occurs worldwide and varies in severity from mild respiratory disease to severe outbreaks with mortality as high as 70% [1]. Although the exact economic losses due to ILT is not known, the poultry industry of the United States experiences multi-million dollar losses each year as a result of ILTV-induced mortality, delayed growth, and decreased egg production.

Currently, live-attenuated vaccines are used to control ILTV infections. However, these vaccines are not satisfactory. The live-attenuated vaccines, particularly the Chick Embryo Origin (CEO), have several disadvantages: (i) reversion to virulence after bird-to-bird passage [2]; (ii) induction of latent infection [3]; (iii) insufficient attenuation [4]; and (iv) difficulty to distinguish the immune response induced by vaccination from that by field infection [5]. Therefore, there is a need to develop a safer vaccine to control and possibly eradicate ILT. A recombinant viral vector vaccine incorporating gene(s) for ILTV immunogens could fulfill these requirements. However, the development of such a vaccine requires knowledge of the protective antigens of ILTV.

It has been shown that vaccination with herpes virus envelope glycoproteins can induce protective immunity [6]. There are eleven glycoproteins in the envelope of ILTV [7]. A subunit vaccine containing glycoprotein B (gB) protected 100% of chickens against clinical disease and also against viral replication [8, 9], indicating that it is a major protective immunogen of ILTV. However, the role of other major envelope glycoproteins, such as gC and gD, in protection and immunity has not been evaluated.

In this study, we have used Newcastle disease virus (NDV) as a vector to express the immunogenic protein(s) of ILTV. We hypothesize that recombinant NDV (rNDV) expressing the envelope glycoprotein(s) of ILTV would provide complete protection to chickens from virulent ILTV challenge. Several characteristics of NDV suggest that a rNDV expressing envelope glycoprotein(s) of ILTV would be a very good vaccine candidate. NDV strain LaSota is widely used as a vaccine with a proven track record of efficacy and safety. NDV naturally enters via the respiratory tract and therefore, elicits mucosal immunity at the respiratory tract, the portal of entry for ILTV. NDV elicits strong humoral and cellular immune responses. NDV replicates in the cytoplasm of infected cells and does not undergo genetic recombination, which makes the expression vector stable and safe. NDV is a relatively small virus with six genes; therefore, chances are better for a lack of significant immune competition between engineered ILTV antigen and NDV-specific antigens.

Our laboratory has developed a reverse genetics system to produce infectious rNDV strain LaSota from cloned cDNAs [10]. This system has been successfully used to express several foreign antigens [11-18]. Therefore, the reverse genetics system for NDV and the expertise are available in our laboratory to express the ILTV genes.

In the present study, we have investigated the individual contributions of three important envelope glycoproteins of ILTV (gB, gC, and gD) in protection and immunity. In herpes simplex virus, gC mediates attachment of virions to cells, while gB and gD are essential for attachment and entry of virus into the cell [19-21]. The gB has been shown to be a major protective immunogen of ILTV [22], but the roles of gC and gD have not been determined. In closely related human and animal herpes viruses, gB, gC, and gD have been shown to be the major protective immunogens [6, 23, 24]. Therefore, the overall objective of this study is to determine the roles of these three ILTV glycoproteins in protection and immunity.

1.2 Research objectives

The specific objectives in the present study on NDV vectored ILTV vaccines are:

1. Constructions of recombinant NDV strain LaSota cDNAs containing gB, gC, and gD genes of ILTV.
2. Recovery and characterization of recombinant NDVs containing gB, gC and gD genes of ILTV.
3. Evaluation of immunogenicity and protective efficacy of recombinant NDVs expressing ILTV glycoproteins in chickens.

Chapter 2: Literature review

2.1 Infectious laryngotracheitis virus (ILTV)

ILTV is a member of the genus Iltovirus within the subfamily alphaherpesvirinae of the family herpesviridae [1, 25]. The virion varies in size from 150-250nm. A mature ILTV particle consists of a core containing a linear double-stranded DNA, an icosahedral capsid containing 162 capsomers, an amorphous material that surrounds the capsid and is designated the tegument, and an envelope containing viral glycoproteins [25]. The ILTV genome sequence has been assembled from sequenced regions of different strains resulting in a total of 148,687 nucleotides with 77 open reading frames (ORF) predicted to be translated into 73 different proteins [26]. However, the genome size of ILTV may vary depending on strains and/or passage history [27]. The ILTV DNA has a guanine plus cytosine ratio of 45% [25]. The genome is arranged into unique long (UL) and unique short (US) regions bounded by terminal repeats (TR) and inverted repeats (IR) (Fig.1.).

In virions, the ILTV genome exists predominantly in two different isometric forms. In 50% of virion genomes, the US region is directed in one orientation and it exists in the opposite orientation in the remaining 50% of the genomes. In ILTV, the UL region is 113kb and the US region is 13kb. ILTV strains are antigenically homogenous based on virus neutralization and cross protection studies [28]. However, minor antigenic differences have been reported among strains [29]

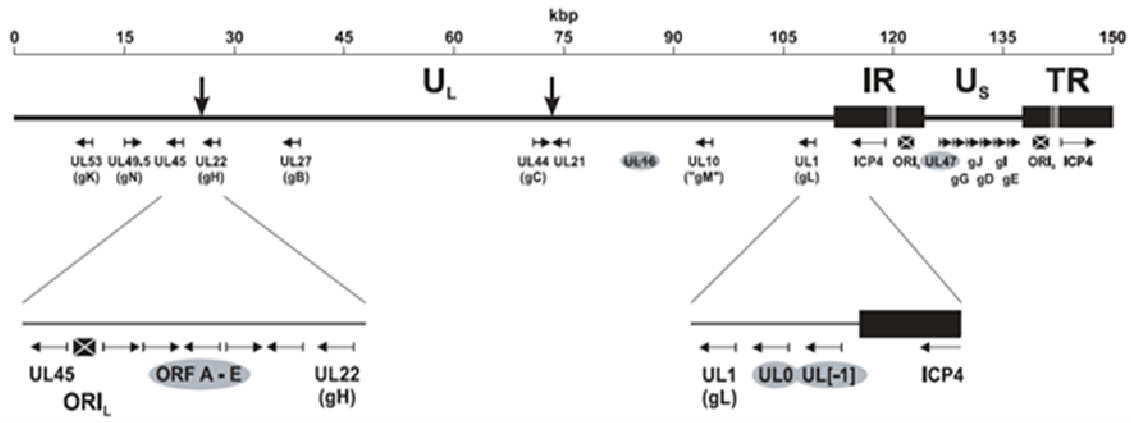

Fig.1. Map of the double-stranded DNA genome of ILTV (150 kbp) [25].

In herpesviruses, the glycoproteins on the surface of envelope are the primary inducers and targets of the humoral and cell mediated immune (CMI) responses [6]. Eleven glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM) have been identified on the envelope of ILTV and most of these glycoproteins are conserved in Alphaherpesviruses [25]. A subunit ILTV vaccine made of a 205 KDa complex containing gB protected 100% of chickens [8, 9], indicating that gB is a major protective immunogen. The role of other ILTV glycoproteins in immunity and protection has not been evaluated. However, ILTV glycoproteins have homologous glycoproteins in other herpesviruses where their functions are known. For example, in herpes simplex virus (HSV) it was found gB, gC, gD, gE and gI induced highest neutralizing titers and protective immunity [6]. In Pseudorabies virus (Prv), gC has been identified as a major target of neutralizing antibody [30-33] and T-cell mediated immune responses [34, 35]. Neutralizing antibodies have also been isolated that are directed against gB and gD of Prv [32, 33].

In bovine herpesvirus-1 (BHV-1), gB, gC and gD are effective immunogens and can protect calves from virulent field challenge [36-40]. These results suggest that any one of the three ILTV glycoproteins (gB, gC and gD) may be useful as a subunit ILTV vaccine individually or in combination.

2.2 ILTV pathogenicity and immunity

ILTV has a very narrow host range, not only in-vitro, but also in-vivo. ILTV can be propagated in embryonated chicken eggs and in primary kidney and liver cell cultures of chicken or chicken embryos [41, 42]. ILTV primarily infects chickens but can also infect pheasants and peafowl [1, 43]. Natural infection of turkeys is rare [44], unless experimentally infected [45]. ILTV infection predominantly affects the mucosa and submucosa of the upper respiratory tract, but conjunctivitis is also frequently observed. Depending on virus dose and strain, the incubation period can vary from 2 to 12 days. Like other herpesviruses, ILTV can establish lifelong latent infection in the central nervous system [46, 47], where the ILTV genome could be detected predominantly within the trigeminal ganglion [48].

ILTV infection induces the production of IgG, IgM and IgA antibodies directed against ILTV proteins. Virus neutralizing antibodies in the serum are first detected within 5-7 days after infection and can remain for a year or more [49]. Mucosal antibodies (IgG and IgA) are detected in tracheal secretion from approximately 7 days after infection [49, 50]. CMI has not been studied directly because of the complexity of requirements in conducting CMI experiments in chickens, but is thought to play an important role in protection and immunity [51].

2.3 ILTV vaccine development

Vaccination with live-attenuated vaccines has been the principal method for prevention and control of ILT [1]. Current live attenuated ILTV vaccines are prepared either by serial passage in chicken embryo or by serial passage in tissue culture. These vaccines confer long lasting protection against pathogenic ILTV strains [1]. However, these vaccines are empirically made and many of them possess residual virulence, which can further increase during animal passage [52, 53]. It is believed that most ILT outbreaks in the USA are caused by vaccine-related isolates that persist in the field [54]. Furthermore, ILT vaccine virus may become latent and reactivation of which can lead to ILT outbreak [3].

Several strategies have been attempted for the development of a vaccine that has reduced potential for clinical disease and latency. One of the strategies has been creation of ILTV mutants for use as live-virus vaccines. Although attempts have been made to engineer stable attenuated ILTV mutants, these attempts have not been successful due to lack of infectious full length clones of ILTV genome and availability of suitable cell lines for in-vitro replication and manipulations. Nevertheless, ILTV recombinants with deletion of at least 14 different genes have been generated by cotransfection of chicken cells with viral DNA and transfer plasmids [7]. A thymidine kinase (TK) gene-deleted ILTV expressing the green fluorescent protein gene was shown to be nonpathogenic, but showed protection against virulent virus challenge similar to commercial vaccine [55]. One of the disadvantages of using recombinant ILTV as a vaccine is that it is unlikely to completely eliminate latency by this method and

that the gene-deleted vaccine virus could become virulent after recombination with wild-type virus, as has been demonstrated for Prv vaccine [56, 57].

Live virus-vectored vaccines for immunization of chickens against ILTV have been developed [22, 58]. A recombinant herpesvirus of turkey (HVT) containing ILTV gene produced protection against virulent ILTV challenge similar to that induced by a commercial vaccine (CEO) [59]. This vaccine is commercially available and may be administered to one-day old chickens subcutaneously or in-ovo to 18-day old chicken embryos. A recombinant fowl pox virus containing ILTV-gB gene also produced protection against virulent ILTV challenge [22, 58].

Another fowl pox virus recombinant containing ILTV- gB and UL-32 gene has been constructed and this virus also provided adequate immunity against virulent ILTV challenge [60]. A recombinant fowl pox vaccine with ILTV genes is commercially available in the United States. This vaccine is recommended for use via wing-web administration in healthy one-week old chicks or in-ovo in 18-day old chicken embryos.

All studies conducted to date suggest that a virus-vectored ILTV vaccine will be most effective for prevention and control of ILT. A vectored-vaccine will be safe and not lead to reversion to virulence or establishment of latency. Several viruses such as fowlpox, adenovirus, adeno-associated virus, NDV and HVT are available for use as vaccine vectors. But each viral vector has its own advantages and disadvantages for use as a vaccine vector for ILTV. Some of the desirable properties for an ILTV vaccine vector are: i) the vector must have a proven track record of safety; ii) ability to vaccinate one-day-old chicks; iii) ability to induce mucosal immunity at the

respiratory tract; iv) expression of the ILTV antigen in proper conformation; and v) expression of the ILTV antigen in a quantity sufficient for induction of a strong neutralizing antibody and CMI responses.

There are several features that make rNDV an attractive vector for ILTV vaccine. Avirulent NDV strains have been widely used as vaccines for more than 50 years with a proven record of safety and efficacy. NDV is a respiratory pathogen and efficiently induces mucosal immunity at the respiratory tract, which is also the site of replication for ILTV. NDV induces strong systemic IgG antibody and CMI responses [15]. NDV replicates in the cytoplasm and does not integrate into the host genome obviating concerns about cellular transformation. In the United States, NDV vaccines are applied by course-spraying of day-old chicks. Therefore, an NDV vectored ILTV vaccine can be applied to a large number of one-day old birds in a short time.

2.4 Newcastle disease virus (NDV)

NDV causes serious respiratory and neurological disease in all species of birds. Newcastle disease varies in degree of severity, ranging from an inapparent infection to severe disease causing 100% mortality. NDV strains are categorized into three main pathotypes, depending upon the severity of the disease produced in chickens [61]. Lentogenic strains do not cause disease and are considered avirulent. Viruses of intermediate virulence are termed “mesogenic”, while virulent viruses that cause high mortality are termed “velogenic”. Several lentogenic and mesogenic strains of NDV are currently used as live attenuated vaccines in chickens [62].

NDV is a member of the genus Avulavirus of the family Paramyxoviridae [63]. The genome of NDV is a single strand negative-sense RNA consisting of 15, 186

nucleotides [64, 65] (Fig.2.). The genomic RNA contains six genes, which encode at least seven proteins [65, 66]. Three proteins constitute the nucleocapsid- the nucleocapsidprotein (N), the phosphoprotein (P), and the large polymerase protein (L). Two proteins form the external envelope spikes, namely, the F and HN proteins. The matrix protein (M) forms the inner layer of the virion. The genomic RNA is tightly bound by the N protein and with the P and L proteins form the functional nucleocapsid within which reside the viral transcriptive and replicative activities. In common with several other paramyxoviruses, NDV produces a seventh protein (V) by editing of the P gene [65, 67].

NDV follows the general scheme of transcription and replication of other non- segmented negative-strand RNA viruses [68]. The polymerase enters the genome at a promoter in the 3' extragenic leader region and proceeds along the entire length by a sequential stop-start mechanism during which the polymerase remains template-bound and is guided by short conserved gene-start (10 nt) and gene-end (10 nt) signals (Fig.2). This generates a free leader RNA and six non-overlapping subgenomic mRNAs. However, 20-30% of the polymerase fails to reinitiate transcription of the downstream genes. Thus, the abundance of the various mRNAs decreases as the distance from the promoter increases. The genes are separated by short intergenic regions (1-47 nucleotides), which are not copied into the individual mRNAs. The 3' terminus (leader) and the 5' terminus (trailer) of the genomic RNA contain cis-acting sequences important for replication, transcription, and packaging of viral RNA [68]. RNA replication occurs when the polymerase switches to a read through mode in which the transcription signals are ignored. This produces a complete

encapsulated positive-sense replicative intermediate that serves as the template for progeny genomes.

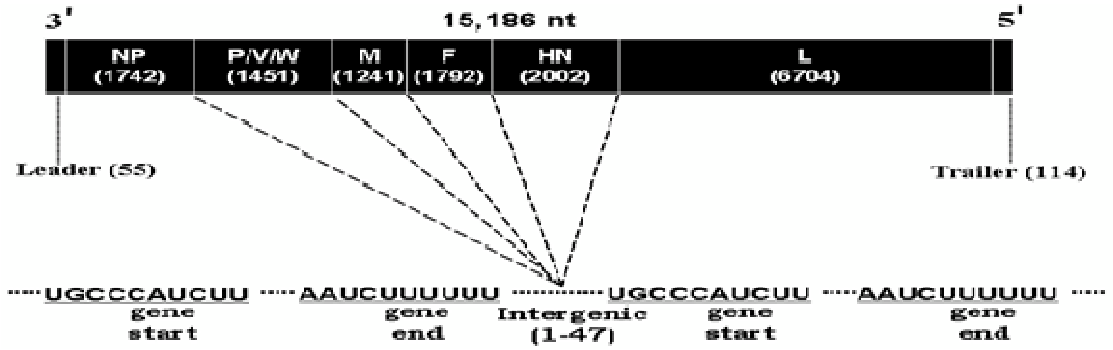

Fig. 2. Genomic map of NDV.

2.5 Generation of recombinant NDV from plasmid DNA

The genomic or antigenomic RNA of negative-stranded RNA viruses is not infectious alone. Other components of the ribonucleoprotein (RNP) complex, namely, N. P and L proteins, are essential to transcribe the genomic RNA into mRNAs resulting in viral protein production and initiation of infection. Therefore, the current technique of genetic manipulation of negative-strand RNA viruses by reverse genetics involves co-transfection into permissive cells with plasmids expressing full-length RNA and N, P and L proteins, all under the control of the T7 promoter. This results in reconstitution of the RNP complex inside the cell and recovery of the infectious virus. The source of T7 RNA polymerase is either a recombinant vaccinia virus expressing the T7 RNA polymerase [69, 70], or a cell line constitutively expressing the T7 RNA polymerase [71]. Using this approach, rabies virus was the first non-segmented, negative- strand RNA

virus to be recovered entirely from cDNA [72]. Since then, several non-segmented, negative-strand RNA viruses have been recovered from cloned cDNAs [73-77].

We have applied these principles to recover lentogenic NDV strain LaSota [10], mesogenic NDV strain Beaudette C [64] and velogenic NDV strain Texas GB [78] from cloned cDNAs. The NDV-derived cDNA sequence was assembled from RT-PCR products obtained using specific primers and viral RNA of the NDV strain. This cDNA (15,186nt) was flanked by a T7 promoter and by the hepatitis delta virus ribozyme sequence (Fig. 3).

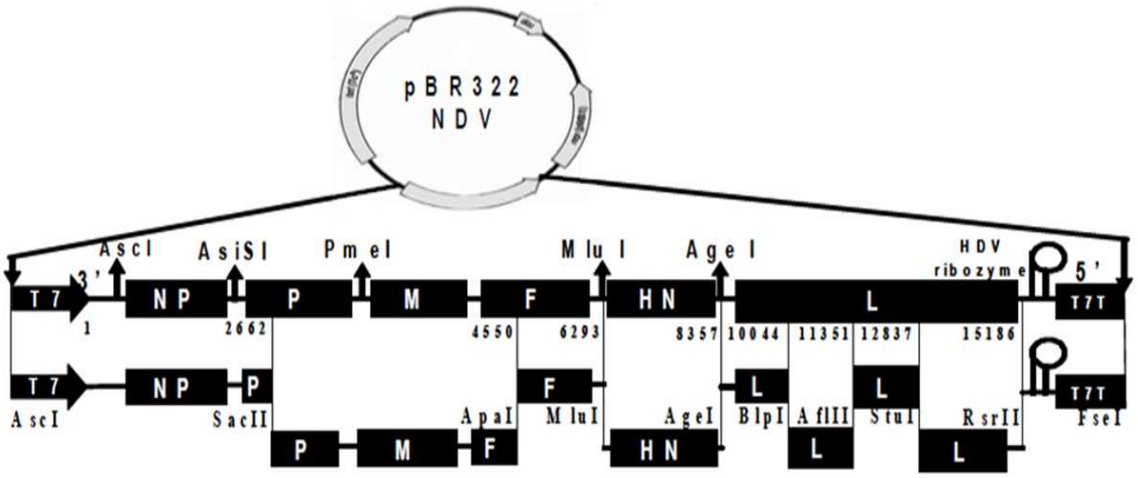

Fig. 3. Assemblies of full-length antigenome of NDV strain LaSota in pBR322. RT-PCR fragments of NDV antigenome was assembled between the T7 promoter and hepatitis delta virus ribozyme as shown in a sequential manner. During construction of the full-length NDV cDNAs, several genetic markers were introduced into the intergenic regions for identification of rNDVs and to facilitate insertion of foreign genes.

2.6 Generation of rNDV expressing foreign genes

The modular organization of NDV genome makes it easy to insert foreign genes. The introduction of a foreign gene flanked by viral transcription gene-start and gene-end signals results in transcription of an additional mRNA (Fig. 4).

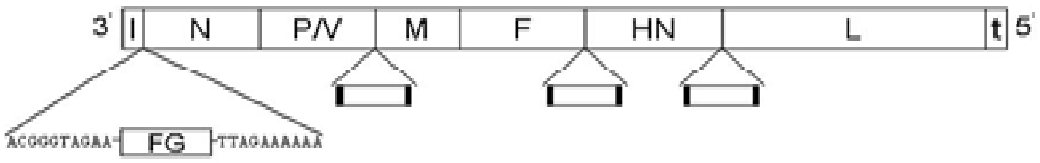

Fig. 4. Schematic diagram of insertion of a foreign gene into NDV antigenome. Empty boxes represent insertion sites used in our laboratory.

We also include an optimal Kozak translation sequence just upstream of the ATG. For optimal replication, the final NDV genome length is adjusted so that it is divisible by six [79], a requirement known as the “rule of six” that is common to most paramyxoviruses.

We have been successful in expressing several viral and bacterial proteins using recombinant NDV as a vector [11-18]. We have also recovered rNDVs expressing two foreign genes. In all cases, it was demonstrated that the rNDV expressed the foreign protein to high levels, and the expression of foreign proteins was stable for many passages (at least 10). Although the upper limit for insertion of a foreign gene in NDV vector is not known, we have been able to insert the S gene of SARS-CoV (3·9kb) into the rNDV genome [16]. The rNDV- SARS/S was recovered without much difficulty and grows well in vitro.

Chapter 3: Materials and Methods

3.1 Cells and Viruses

Human epidermoid carcinoma (HEp-2), chicken embryo fibroblast (DF1), and Vero cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). HEp-2, DF1, and vero cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in DMEM with 5% FBS. Chicken embryo liver cells (CELi) were harvested from 11 -12 day old specific pathogen free embryonated chicken eggs by conventional trypsin disaggregation method and were grown in Eagle's minimal essential medium (EMEM) containing 10% FBS. The chicken-embryo-origin ILTV vaccine Trachivax was obtained from the Schering-Plough Animal Health Corp, Millsboro, DE. The USDA challenge strain of ILTV was obtained from the National Veterinary Services Laboratory, Ames, IA, USA. The USDA ILTV challenge strain was propagated on monolayers of chicken embryo liver cells. Recombinant NDV strains were grown in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The modified vaccinia virus Ankara strain expressing T7 RNA polymerase was grown in primary chicken embryo fibroblast cells.

3.2 Isolation of ILTV DNA

The chicken-embryo-origin ILTV vaccine Trachivax (Schering- Plough Animal Health Corp, Millsboro, DE) was used as the source for ILTV DNA. Briefly, ILTV was propagated in chick embryo liver (CELi) cells. Virus cultures was harvested when moderate level of cytopathic effects (CPE) characterized by rounding and syncytia formation was observed. The virus- infected cultures was harvested after two freeze and

thaw cycles. Cellular debris was removed by slow centrifugation at 1000Xg for 10 min at 4OC. Virus was harvested by pelleting the clarified supernatant through 30% sucrose cushion in phosphate-buffered saline at 100,000 g for 60 min. The virus pellet was suspended in 0.05 M Tris–HCl, 0.15 M NaCl, 10 mM EDTA (pH 8.0) and applied to a 20 to 50% potassium-sodium tartrate discontinuous gradient and centrifuged at 100,000 g for 90 min. After centrifugation, the virus band was collected, diluted in PBS, and pelleted at 75,000 g for 60 min. Viral DNA was purified according to the method of Summers et al. [82]. Briefly, purified virus was suspended in extraction buffer (0.1M Tris–HCl, 0.15M NaCl, 0.1 M EDTA, 0.1 M KCl, pH 7.5) containing 45 µg of proteinase K per ml and incubated at 50˚C for 1 h. Sarkosyl was added to a final concentration of 1%, and the sample was incubated at 50˚C for an additional 1 h. The sample was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. The DNA pellet was resuspended in 0.05 M Tris–HCl, 10 mM EDTA, pH 8.0.

3.3 Modification of ILTV gB gene

ILTV gB gene is 2652 nucleotides in length and has a guanine plus cytosine ratio of 44.53%. Detailed scanning of ILTVB gB gene demonstrated sequence similarities to NDV strain LaSota gene end signals at nucleotide positions(ORF positions) 566-573 and 889-894 which contains poly A tail consisting of more than six repeated adenine bases. These signals could be potentially read as gene ends by viral RNA polymerase leading to premature termination of transcription. Therefore, we have modified the gB gene at above said nucleotide positions by overlapping PCR methodology and without altering the amino acid sequence of the encoded protein. The wild type and modified gB with the

modified nucleotides and their positions is shown in the following table (Table.1). Modified gB was used for subsequent cloning into rNDV vector.

Mutagenesis at 567,570 and 573 ORF positions of ILTV gB gene **5'-AAT GAT GAA GCA GAa AAa AAa TTG CCC CTG GTT CCA TCA CTG-3'** **5'-AAT GAT GAA GCA Gag AAg AAg TTG CCC CTG GTT CCA TCA CTG-3'** **(Nucleotides at positions 567,570 and 573 in wild type gB (first row) are shown in lower case and their modifications are shown in modified gB (second row) in lower case red).**
Mutagenesis at 891 and 894 ORF positions of ILTV gB gene **5'-ATA AGA CCC CCT AAa AAa AGA AAC TTT CTA ACA GAT GAA -3'** **5'-ATA AGA CCC CCT AAg AAg AGA AAC TTT CTA ACA GAT GAA -3'** **(Nucleotides at positions 891 and 894 in wild type gB (first row) are shown in lower case and their modifications are shown in modified gB (second row) in lower case red).**

Table 1. Modification of ILTV gB gene

3.4 Cloning of ILTV gB, gC and gD genes

Three sets of PCR primers were used to amplify the ORF of gB, gC and gD genes of ILTV. Sequences of three primer sets are gB (forward), 5'GATCGTTTAAACTTAGAAAAAATACGGGTAGAAGGCCACCatgcaatcctacatcgcg 3'; gB (reverse), 5' GATCGTTTAAACttattcgtcttcgctttcttctgc3'; gC

(forward),5’GATCGTTTAAACTTAGAAAAAATACGGGTAGAACGCCGCCACCatg cagcatcagagtactgcg3’;gC(reverse),5’GATCGTTTAAACttatgttgtcttccagcaccatg3’;gD(for ward),5’GATCGTTTAAACTTAGAAAAAATACGGGTAGAACGCCACCatggaccgcca tttatttttgag3’;gD(reverse)5’GGCCGTTTAAACttagctacgcgcgcattttacg 3’. All primers contains PmeI sites (italicized), the NDV gene end and gene start transcriptional signals (underlined), the T intergenic nucleotide (boldface), additional nucleotide in order to maintain the genome length as a multiple of six (italicized and bold), and a six-nucleotide Kozak sequence for efficient translation (bold, underlined). The ILTV-specific sequence is in small case. PCR was performed using 100 ng of pre-denatured viral DNA, 50 pmol of each primer, 2 × GC buffer I containing Mg2+, 200 μM dNTPs, 0.5 units of TaKaRa LA Taq™ polymerase (Takara Bio USA, Madison, WI). PCR will be carried out for 30 cycles under the following conditions; 1 min at 94OC for denaturation of the template, 1 min at 55OC for primer annealing and 3 min at 72OC for chain extension. After amplification, the 2652, 1245 and 1134 base pair products were digested with PmeI and cloned into pCR 2.1-TOPO vector (Invitrogen). The integrity of the gB, gC and gD genes was confirmed by sequence analysis.

3.5 Construction of rNDV cDNAs containing gB, gC and gD genes of ILTV

In the present study, we have inserted additional transcriptional units containing ILTV gB, gC and gD genes between the P and M genes of NDV. Based on previous studies from our laboratory, large size inserts can be placed between the P and M genes without affecting virus replication [11]. The NDV plasmid used in this study is a full-length cDNA of the antigenomic RNA of NDV strain LaSota that was modified to contain a unique PmeI restriction enzyme site between the P and M genes [12].

The TOPO vectors bearing the glycoproteins (gB, gC and gD) gene of ILTV were digested with PmeI and cloned at the unique PmeI site between P and M genes of full-length NDV plasmid to yield pNDV-gB, pNDV-gC and pNDV-gD (Fig. 5). The resulting plasmids were sequenced to confirm orientation and the absence of adventitious mutations.

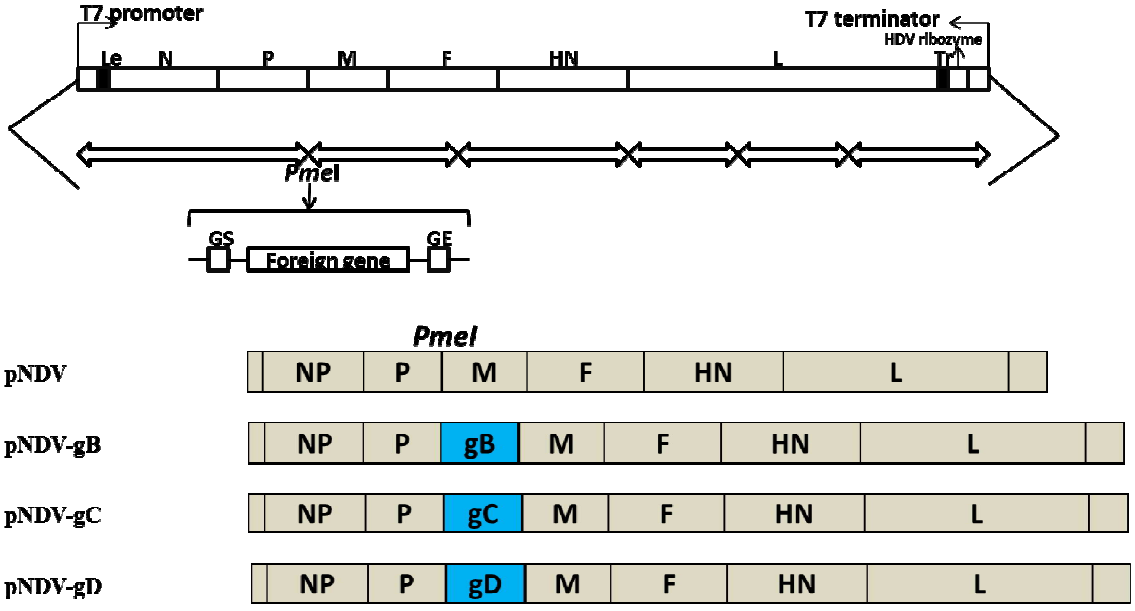

Fig. 5 Construction of NDV cDNAs containing ILTV genes

3.6 Antisera production in rabbits

The anti- ILTV antiserum was raised in rabbits against the synthetic peptides of gB, gC, and gD proteins of ILTV. Synthetic peptides of ILTV gB, gC and gD proteins were obtained from GenScript USA Inc., Piscataway, NJ, USA. Briefly, rabbits were injected with the synthetic peptides of gB, gC and gD initially, and followed by two booster doses

at 14 day interval time. Synthetic peptides were homogenized with the Freund's complete adjuvant in equal proportion for the initial dose, but for the booster doses mixture of synthetic peptides and Freund's incomplete adjuvant in equal proportion was used. The rabbits were bled after the final booster and the serum samples were collected. The specificity of the antiserum was determined by Western blot analysis.

3.7 Transfection

The recombinant viruses were recovered using the reverse genetics techniques as depicted in Fig. 6. Briefly, HEp-2 cells were used for transfection experiments because these cells are permissive for NDV, transfect efficiently, and are resistant to CPE of vaccinia virus strain MVA expressing the T7 RNA polymerase. A mixture of three plasmids containing NDV N, P and L (0·4µg, 0·3µg, and 0·2µg per well, respectively) and a fourth plasmid encoding the full- length NDV antigenome carrying the ILTV sequences (4.0µg) were transfected with Lipofectamine (Invitrogen) and infected with MVA. Four hours after transfection, the cells were washed twice and replaced with medium containing acetyl-trypsin (1µg/ml). Forty-eight hours after transfection, the cells were scraped, sonicated and centrifuged at 1000 xg for 5 min. The clarified supernatant was used to infect HEp-2 cells. After two passages in HEp-2 cells, 100µl clarified supernatant was used to inoculate into the allantoic cavity of 10-day-old embryonated chicken eggs. The allantoic fluid was harvested 4 days later and tested for HA.

3.8 Reverse transcription PCR (RT-PCR) and sequence analysis of cloned ILTV genes

Total RNAs were isolated from the rNDVs infected allantoic fluid in the 9-day-old SPF chicken embryo eggs using RNeasy Mini Kit (Qiagen) according to

manufacturer's recommendations. Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen). The positive-sense primer used for RT reaction was NDV P gene 3120(P3120F) Forward primer, 5' CGGAATCTGCACCGAGTT 3' for all rNDV's carrying ILTV genes. For rNDV-gB, the generated cDNA product was PCR amplified using primers, P3120F and gB 500R 5' CATTCTCCCGATCGATCAAT 3'. For rNDV-gC, the primers, P3120F (+) and gC 500R (-) 5' GTTGTAGGTGGTCTTGCCAA 3', were used for the PCR amplification. For rNDV-gD, the primers, P3120F (+) and gD 500R (-) 5' AGCAAGTATTGGCCAGAGAG 3', were used for the PCR amplification. The RT-PCR products were separated on 1% agarose gel, and the sequences were confirmed by sequencing.

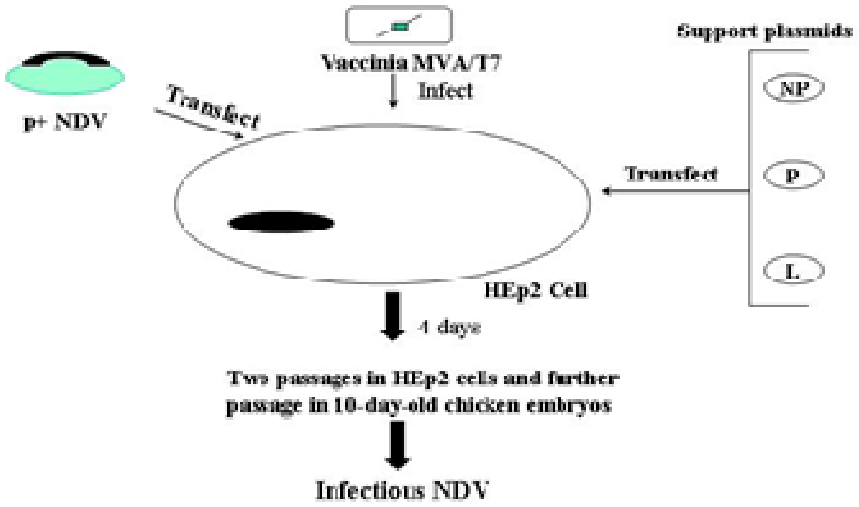

Fig. 6. Method of transfection and rescue of recombinant NDV-ILTV (The figure was modified from Krishnamurthy et al.,2000 (64)).

3.8 Western blot analysis

The expression of gB, gC and gD proteins by rNDVs was examined by Western blot analysis. Briefly, DF1 cells were infected with rNDV's at an MOI of 0.1 and were harvested at 48 h post- infection. Further, DF-1 cells were lysed using RIPA-PMSF buffer and analyzed by Western blot using peptide antisera against gB, gC and gD proteins. To examine the incorporation of ILTV glycoproteins into NDV particles, Western blot analysis was carried out using purified virus and peptide antisera against gB, gC and gD proteins.

3.9 Growth kinetics of rNDV's expressing ILTV gB, gC and gD

The growth kinetics of rNDV's expressing ILTV gB, gC and gD were determined by multicycle growth curves in DF1 cells. Briefly, DF1 cells were infected with viruses at a MOI of 0.01 PFU and the cell culture supernatant samples were collected at 12-hour intervals until 60 hours post-infection. The titers of virus in the samples were quantified by plaque assay in DF1 cells. All plaque assays were performed in 12-well plates. Briefly, supernatants collected from the virus infected DF-1 cell samples were serially diluted and each dilution (200 μL) was infected to 12 well plate DF-1 cells in duplicates. After 1 h adsorption, cells were covered with DMEM containing 2% FBS, 0.8% methylcellulose, and incubated at 370C in 5% CO2. Four days later, the cells were fixed with methanol and stained with crystal violet for enumeration of plaques.

3.10 Mean death time of rNDV's expressing ILTV gB, gC and gD

Mean death time was determined by inoculating the diluted virus (10-6 to 10-9) into 9-days-old embryonated eggs (5 eggs for each diluent). The viability of embryo in the infected eggs was evaluated every 8 h up to 144 h. The time of each embryo death was

recorded and the highest dilution at which all five embryos died was considered as the minimum lethal dose. The MDT was calculated as the mean time in hours taken by the minimum lethal dose to cause the death of all the embryos.

3.11 Immunofluorescence assay

The expression of ILTV proteins by the recombinant viruses was examined in vero cells by immunofluorescence assay. Briefly, confluent monolayers of vero cells on 4 well Lab-Tek chamber slides were infected with the recombinant viruses at a multiplicity of infection (MOI) of 0.1. After 24 h, the infected or control cells were washed with phosphate buffered saline (PBS) and either fixed with 4% paraformaldehyde for 20 min at room temperature for detection of surface antigen, or fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 10 min for detection of total antigen. After further washing with PBS, the cells were incubated for 30 min with 3% normal goat serum to block nonspecific binding sites and incubated for 1 h with 1:50 dilution of respective rabbit polyclonal anti-ILTV antiserum. The cells were rinsed with PBS and incubated with 1:1000 dilution of Alexa Fluor 488 conjugated goat anti-rabbit immunoglobulin G antibody (Invitrogen, Carlsbad, CA) for 45 min. The cells were washed with PBS and analyzed with a fluorescent microscope.

3.12 Evaluation of the genetic stability of rNDV's containing ILTV glycoprotein genes

The genetic stability of rNDV's containing ILTV glycoprotein genes was evaluated by 10 serial passages in 9- day old SPF eggs and 10 serial passages in DF-1 cells. Virus preparations were made from the 10th passage allantoic fluid and 10th passage infected

DF-1 cell supernatant and subjected to sequence analysis to determine the genetic integrity of the cloned ILTV genes.

3.13 Immunization and Challenge experiments in chickens

The immunogenicity and protective efficacy of the recombinant viruses against virulent ILTV were evaluated in specific pathogen free (SPF) chickens obtained from Charles River Laboratories, Wilmington, MA, USA. All of the animals used in this study were housed in isolator cages in our biosafety level 2+ facility and were cared for in accordance with established guidelines, and the experimental protocols were performed with the approval of Institutional Animal Care and Use Committee (IACUC) of the University of Maryland and under Animal Welfare Association (AWA) regulations. The challenge experiment with virulent NDV strain Texas-GB was carried out in an enhanced BSL3 containment facility certified by the USDA and CDC, with the investigators wearing appropriate protective equipment and compliant with all protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland and under Animal Welfare Association (AWA) regulations.

Immunization experiment

Two-week-old SPF white leghorn chickens were assigned to 10 groups of 26 chickens each and placed in isolation units maintained under negative pressure (Table.4). A group 1 was remaining unvaccinated and serves later as challenge controls. Group 2 was vaccinated with recombinant NDV strain LaSota vaccine by oculonasal instillation of 106 EID50 of virus. Group 3 was vaccinated with the CEO vaccine Trachivax (Schering-Plough Animal Health Corp, Millsboro, DE) as per manufacturer's recommended dose.

Group	No Chickens	Vaccine virus	Challenge virus
1	26	Challenge control	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
2	26	rNDV LaSota vaccine	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
3	26	Trachivax	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
4	26	rNDV-gB	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
5	26	rNDV-gC	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
6	26	rNDV-gD	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
7	26	rNDV-gB + rNDV-gC	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
8	26	rNDV-gB + rNDV-gD	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
9	26	rNDV-gC + rNDV-gD	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.
10	26	rNDV-gB + rNDV-gC + rNDV-gD	(13 birds) NDV-GB Texas. (13 birds) USDA ILTV.

Table.4. Protocol for evaluating the immunogenicity of rNDVs expressing envelope glycoproteins of ILTV.

Groups 4, 5 and 6 were received 106 EID50 of each rNDVs expressing ILTV gB, ILTV gC and ILTV gD respectively by oculonasal instillation. Group 7 received a mixture of 106 EID50 each of rNDV-gB and rNDV-gC, group 8 received a mixture of 106 EID50 each of rNDV-gB and rNDV-gD, group 9 received a mixture of 106 EID50 each of rNDV-gC and rNDV-gD, and group 10 received a mixture of 106 EID50 each of rNDV-gB, rNDV-gC and rNDV-gD by oculonasal instillation respectively. The chickens were immunized with a single dose of rNDV's in a total volume of 0.2 ml (0.05 ml in each eye and nostril). Three weeks post-immunization, prechallenge serum samples were collected for serum antibody response, and chickens in each group were divided into two subgroups of 13 chickens each, one subgroup was transferred to enhanced BSL3 facility for NDV challenge. The remaining chickens were kept in BSL-2+ facility for virulent ILTV challenge.

Challenge experiment

NDV Challenge

Each bird in all groups (n=13) were challenged by oculonasal route with 104.5 ELD50 of Velogenic NDV strain Texas GB. Birds were observed daily for 2 weeks for clinical signs (death, paralysis, and torticollis) of neurotropic NDV. In order to determine the replication of challenge virus, three chickens from each group were sacrificed on day 3 post challenge. Tissue sample (trachea, lungs, and brain) were collected, homogenized in cell culture medium DMEM containing antibiotics (1gm/10ml) and clarified by centrifugation. The challenge virus titers in organs were determined by plaque assay in DF-1 cells.

ILTV Challenge

Each bird in all groups (n=13) were challenged after 3 weeks of vaccination with 0.2 ml of a USDA ILT challenge virus (NVSL, Ames, IA) intratracheally (106 TCID50/bird). All birds were observed daily for 14 days post challenge for clinical signs of respiratory disease, conjunctivitis and mortality. A daily total clinical sign score was calculated for each group following the scoring system described by Oldoni et al [54].

In order to determine the replication of challenge virus, three chickens from each group were sacrificed on day 4 post challenge. The tracheal tissue was observed for macroscopic lesions and then collected in cell culture medium DMEM (1g/mL) containing antibiotics and homogenized. The challenge virus titers in the homogenates were determined by TCID50 method in chicken embryo liver cells using Reed and Muench method as described previously (86).

The remaining ten chickens in each group were observed daily for 14 days for disease signs and mortality following challenge. Post challenge serum was collected from the surviving birds before they were sacrificed on day 14 post challenge. For detection of latent infection, trigeminal ganglia were removed from all the surviving birds on day 14 post challenge as described by Williams et al [47]. The trigeminal ganglia were homogenized in cell culture medium DMEM containing antibiotics, centrifuged and the supernatant was used for the extraction of ILTV DNA [48]. The DNA was isolated using Qiagen's QIAamp DNA mini kit (250) according to manufacturer protocols. The amplification of the TK gene of ILTV DNA was conducted by nested PCR using primer pairs from published TK gene sequence [84]. The primers used were shown in the following table (Table 5).

TK-I Forward: 5'-ACTTCCGGTGGTGTGCAGTTTTGC-3' TK-I Reverse: 5'-TATCAGCATTGTAGCGCT-3'
TK-II Forward: 5'- AGGCTTCCGGAAAACTTGAATGTC-3' TK-II Reverse: 5'- TCTTGAATTTTAAGAGCG-3'

Table.5. Primers used in Nested PCR for amplification of TK gene from ILTV DNA collected from trigeminal ganglion.

3.14 Scoring of clinical signs

All the birds challenged with USDA strain of ILTV were observed daily for 14 days post challenge for clinical signs of respiratory disease, conjunctivitis and mortality. Clinical signs were scored according to the scoring system described by Oldoni et al [54]. The following procedure is extracted from the Oldoni et al (54). Clinical signs were scored on the basis of breathing patterns, conjunctivitis, the level of depression, and mortality observed per unit per day. A daily total clinical sign score was calculated for each unit. Dyspnea was scored on a scale of 0 (normal breathing), 1 (mild dyspnea and open mouth breathing) and 2 (gasping with an extended neck). The condition of the conjunctiva was scored on a scale of 0 (normal), 1 (swollen and/or partial closure of the eyes) and 2 (complete closure of the eyes). The level of depression was scored on a scale of 0 (normal, or not depressed), 1 (depressed) and 2 (severely depressed). For mortality, the score was 0 (no mortality) and 3 (mortality). Statistical analysis was performed to compare clinical sign scores at different days post inoculation (d.p.i.) within each group.

Multiple comparisons with baseline values were made using a Bonferroni correction to limit the overall type-I error rate to 5%.

3.15 Serological analysis

The antibody levels of serum samples collected from chickens vaccinated with rNDV's were evaluated by hemagglutination inhibition (HI), virus neutralization (VNT) assay, and enzyme-linked immunosorbent assay using standard protocols (86).

NDV specific antibody response in the serum samples collected from the birds was measured by hemagglutination inhibition (HI) assay. For the HI assay, twofold serial dilutions of immunized chicken sera (50 μL) were prepared, and each dilution was combined with 4 HA units of rNDV. Following 1 h of incubation, 50 μL of 1% chicken RBC was added and incubated for 30 min at room temperature, and hemagglutination was scored.

ILTV specific antibody response in the serum samples collected from birds was measured by enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VNT) assays. Serum samples were analyzed by ELISA using the LTELISA kit (ProFLOCK® LT ELISA Kit, Synbiotics Corp., San Diego, CA) following the manufacturer's instructions. ILTV neutralizing antibody titers were also determined by virus neutralization assay performed in 96-well plates with chicken embryo liver cells. Briefly, each serum sample was heated at 560c for 30 minutes to destroy heat-sensitive, non-specific virus inhibitory substances. Six two-fold dilutions in duplicate were performed for each serum in minimum essential medium containing proper antibiotics. Each dilution was mixed with 100 TCID50 of USDA ILTV virus in 96-well plate and incubated for 60 minutes at 370c. After incubation the serum virus mix was transferred

and adsorbed into chicken embryo liver cell monolayers. The plate was incubated for 5 days at 370c. After 5 days of incubation, the plate was examined for the presence of ILTV cytopathic effect and the VN titer was estimated as the reciprocal of the first serum dilution were ILTV cytopathic effect was observed.

3.16 Statistical analysis

Statistically significant differences in data from serological analysis of different immunized chicken groups were evaluated by one-way analysis of variance (ANOVA) with the use of Prism 5.0 (Graph Pad Software Inc., San Diego, CA) at a significance level of $P < 0.05$.

Chapter 4: Results

4.1 Construction of rNDV cDNAs containing gB, gC and gD genes of ILTV

The recombinant lentogenic NDV strain LaSota containing a unique PmeI site between the P and M genes was used as a vector to express the ILTV glycoprotein from an added gene. The ILTV gB, gC and gD genes were amplified by PCR from genomic DNA extracted from purified ILTV virions. ILTV gB gene appeared to possess sequence similarities to NDV strain LaSota gene end signals at nucleotide positions(ORF positions) 566-573 and 889-894 which contains poly A tail consisting of more than six repeated adenine bases. These signals could potentially leads to the premature termination of transcription by viral RNA polymerase. Therefore, we have modified the gB gene at above said nucleotide positions by overlapping PCR and the modified gB was used for subsequent cloning into rNDV vector. The gB, gC and gD ORF's were placed under the control of NDV transcriptional signals and inserted at the PmeI site between the P and M genes in the NDV vector. The transcription cassette was designed to maintain the rule of six, whereby the genome nucleotide length must be an even multiple of six in order to be efficiently replicated. A Kozak sequence was inserted before the start codon of the gB, gC and gD gene ORF to provide for efficient translation. The resulting plasmids were designated as pNDV-gB, pNDV-gC and pNDV-gD and their sequencing analysis indicated absence of any adventitious mutations and also confirmed that ILTV genes were cloned in proper orientation (data not shown).

4.2 Recovery and characterization of recombinant NDVs containing gB, gC and gD genes of ILTV

4.2.1 Antisera production in rabbits

Anti-ILTV antibodies in the rabbit serum were titrated using ILTV-ELISA kit from Synbiotics Corporation, San Diego, California, USA. The sequences of the synthetic peptides used and their respective anti-ILTV antibody titers are shown in the following table (Table 2).

Peptide	Sequence	Anti-ILTV antibody titer after 2nd booster
gB1	CPRGRERRQAAGRRT	Anti-ILTV gB : 6582
gB2	AIGSGAPKEPQIRNC	
gB3	CNLFRRKPRTKEDDY	
gC1	CLEIRGEASQPLPSK	Anti-ILTV gC : 5394
gC2	CTPPEDFEMLRPETR	
gC3	FSDRPLTHEESVKVC	
gD1	CRKKNPSAPDPRPDS	Anti-ILTV gD : 5987
gD2	CEDTEHDDPNSDPDY	
gD3	MISAAKEKEKGGPFC	

Table 2. Synthetic peptides used for the production of rabbit polyclonal antisera and their respective anti-ILTV antibody ELISA titers.

4.2.2 Recovery of rNDVs containing the glycoprotein genes of ILTV and their evaluation of genomic integrity

The recombinant viruses, designated as rNDV-gB, rNDV-gC and rNDV-gD were recovered using the reverse genetics method available in our laboratory. The structure of gB, gC and gD inserts in the genome of these viruses was confirmed by RT-PCR (Figure 7) and nucleotide sequence analysis. All the recombinant viruses were propagated in embryonated chicken eggs and the titers were determined by HA assay. The HA titers of recombinant viruses were 1–2 log2 lower than that of the parental rLasota virus. This result is consistent with previous findings that a moderate attenuation of replication can result from the insertion of a foreign gene (64). To determine the stability of the ILTV genes in the recombinant viruses, the recovered viruses were passaged ten times in embryonated chicken eggs and ten times in chicken embryo fibroblast DF-1 cells. Sequence analysis of the ILTV genes of the resulting virus preparations showed that the integrity of the ILTV genes was preserved and stably maintained even after 10 passages.

Figure 7. RT-PCR analysis of the ILTV genes from recombinant NDVs. Lane 1: Invitrogen 1 Kb+ ladder, Lane 2: RT-PCR product amplified from genomic RNA isolated from rNDV-gB infected allantoic fluid, Lane 3: RT-PCR product amplified from genomic RNA isolated from rNDV-gC infected allantoic fluid and, Lane 4: RT-PCR product amplified genomic RNA isolated from rNDV-gD infected allantoic fluid.

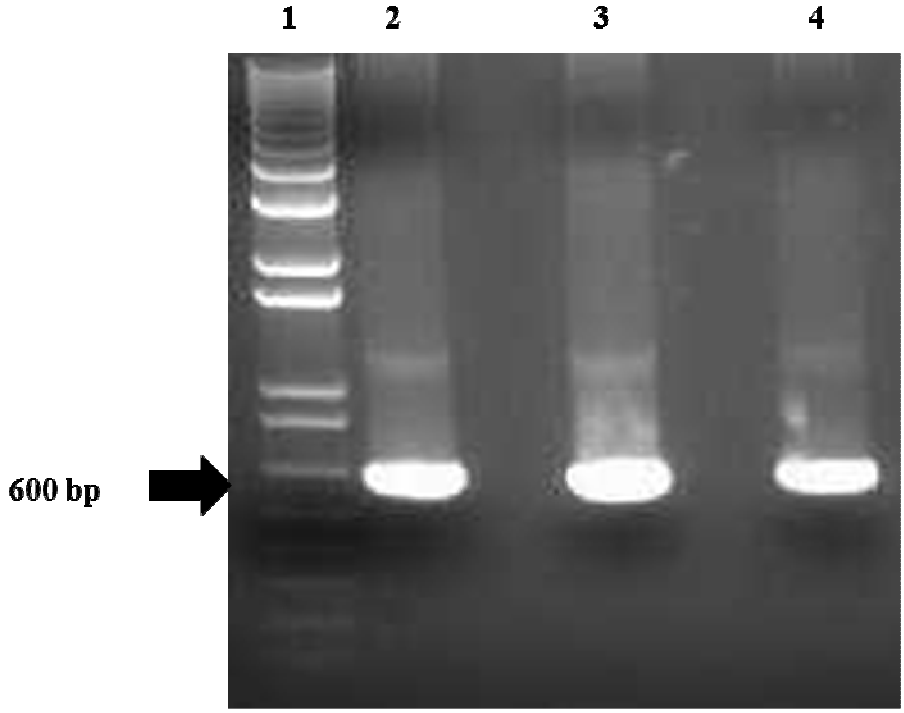

Figure 7. RT-PCR analysis of the ILTV genes from recombinant NDVs.

4.2.3 Expression of ILTV glycoproteins by recombinant viruses

The expression of ILTV glycoproteins in DF-1 cells infected with recombinant viruses was analyzed by western blot using rabbit anti-ILTV peptide antisera (Figure 8). Immunoblot analysis detected two bands in lysates of cells infected with rNDV-gB: these represented (i) dimer form of gB with an apparent molecular weight of 100 kDa and (ii) mature processed form of gB with an apparent molecular weight of 58kDa. Immunoblot analysis detected 60kDa and 42kDa bands in rNDV-gC and rNDV-gD infected DF-1 cell lysates respectively. Surprisingly, only the ILTV glycoprotein C (gC) was found to be incorporated into the envelope of recombinant virus. ILTV glycoprotein B and D were not found to be incorporated into the envelopes of mature virions.

We also used immunofluorescence to determine the intracellular and surface expression of the ILTV glycoproteins in vero cells infected with recombinant viruses. Positive staining was observed for cells infected with rNDV-gB and rNDV-gD using rabbit anti-ILTV antiserum (Figure 9. Immunofluorescence analysis of vero cells infected with rNDV, rNDV-gB and rNDV-gD for intracellular expression and surface expression of ILTV proteins. Vero cells were infected with rNDV (panel B, D, F, and H), rNDV-gB (panel A and C) and rNDV-gD (panel E and G) and probed with rabbit anti-ILTV gB antibodies (panel A, B, C, and D) and rabbit anti-ILTV gD antibodies (panel E, F, G, and H) for intracellular antigen (panel A, B, E, and F) as well as surface expression (panel C, D, G, and H) of ILTV proteins.). We were not able to show the internal and surface expression of ILTV proteins for cells infected with rNDV-gC, although, the rabbit antisera detected ILTV gC protein in DF-1 cells infected with rNDV-gC virus. This observed variation could be partly attributed to the inability of the rabbit anti-ILTV gC antisera to detect conformational epitopes of gC protein in immunofluorescence analysis.

Figure 8. Western blot analysis of the rNDV's expressing ILTV gB, gC and gD proteins. A: 1&4- ILTV control, 2-rNDV-gBPmeI DF1 lysate, 3-rNDV Lasota control, 5-rNDV-gB purified virus, 6-rNDV Lasota purified virus. B:1&4- ILTV control, 2-rNDV-gC DF1 lysate, 3-rNDV Lasota DF1 lysate, 5-rNDV-gC purified virus, 6-rNDV Lasota purified virus. C: 1&4- ILTV control, 2-rNDV-gD DF1 lysate, 3-rNDV Lasota DF1 lysate, 5-rNDV-gD purified virus, 6-rNDV Lasota purified virus.

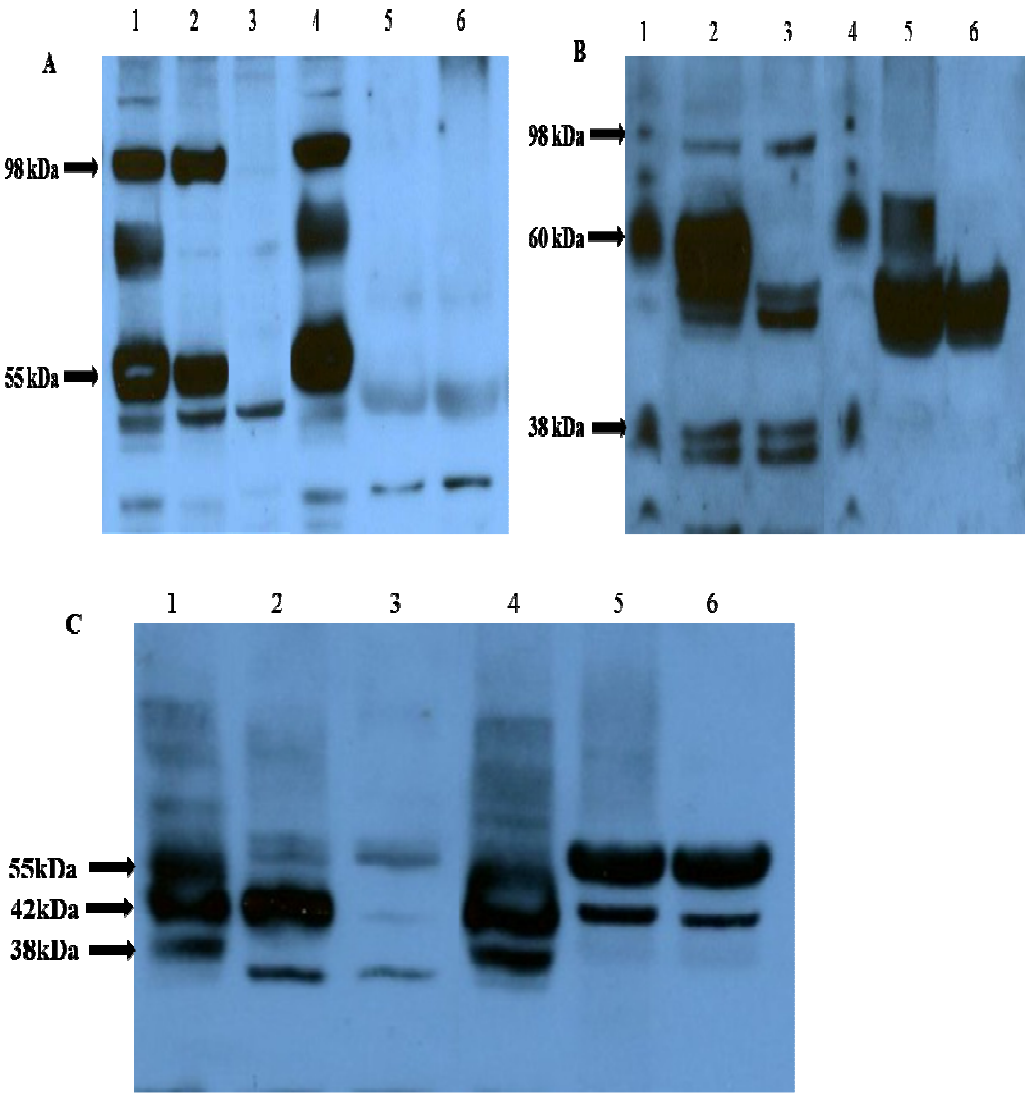

Figure 8. Western blot analysis of the rNDV's expressing ILTV gB, gC and gD proteins.

Figure 9. Immunofluorescence analysis (Immunofluorescence analysis of vero cells infected with rNDV, rNDV-gB and rNDV-gD for intracellular expression and surface expression of ILTV proteins. Vero cells were infected with rNDV (panel B, D, F, and H), rNDV-gB (panel A and C) and rNDV-gD (panel E and G) and probed with rabbit anti-ILTV gB antibodies (panel A, B, C, and D) and rabbit anti-ILTV gD antibodies (panel E, F, G, and H) for intracellular antigen (panel A, B, E, and F) as well as surface expression (panel C, D, G, and H) of ILTV proteins).

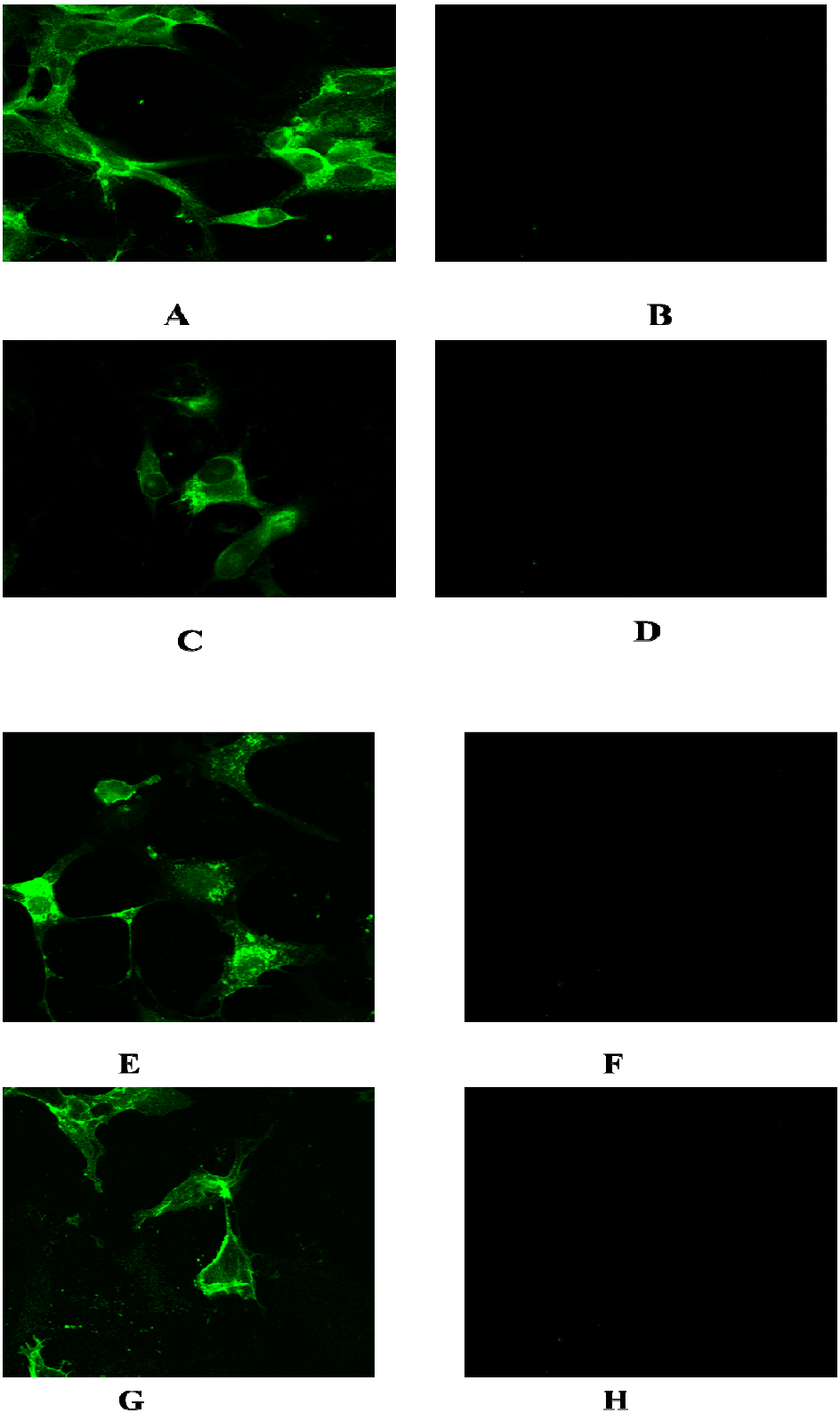

Figure 9. Immunofluorescence analysis

4.2.4 Biological characterization of rNDV's expressing ILTV glycoproteins

The multicycle growth kinetics of rNDV's expressing ILTV glycoproteins were compared with those of the parental rNDV. Our results demonstrated similar kinetics of growth between rNDV and rNDV-gC and rNDV-gD, whereas rNDV-gB grew more slowly and achieved a maximal titer that was approximately one log lower than those of parental rNDV titers (Figure 10). Similar plaque sizes were observed for rNDV and rNDV-gC and rNDV-gD, but a slightly smaller plaque size was observed for rNDV-gB (Figure 11). The pathogenicities of parental rNDV, rNDV-gB, rNDV-gC and rNDV-gD were evaluated by the mean death time (MDT) test in embryonated chicken eggs. The MDTs for these viruses is shown in Table 3. The result of Mean death time test indicates that the addition of ILTV genes did not increase the virulence of NDV strain LaSota vector. Indeed, the presence of the added ILTV genes conferred attenuation to the NDV vector.

Virus	MDT (h)
rLasota	110
rLasota/gB	>120
rLasota/gC	>120
rLasota/gD	117

Table 3. Mean death time of rNDV's expressing ILTV gB, gC and gD proteins

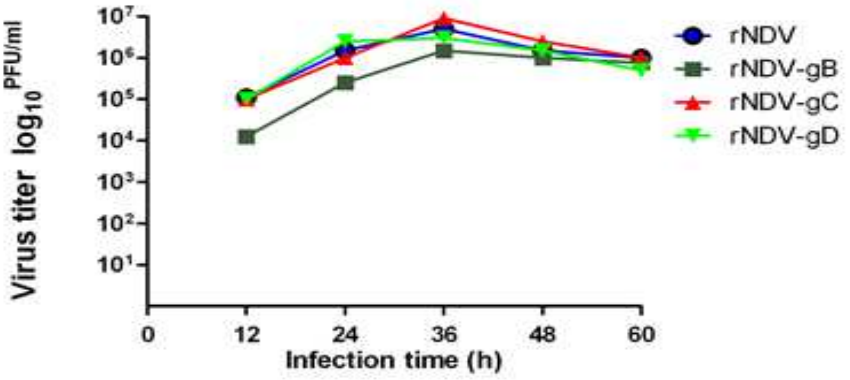

Figure 10. Growth kinetics of rNDV's expressing ILTV gB, gC and gD proteins.

4.2.5 Conclusions

In the present study, we successfully recovered rNDV's containing ILTV genes using reverse genetics system available in our laboratory. The expression of ILTV glycoproteins by recombinant viruses is confirmed by western blot analysis using DF-1 cells and Immunofluorescence using vero cells. The ILTV glycoprotein C (gC) was found to be incorporated into the envelope of recombinant virus. The recombinant viruses were able to replicate invitro in DF-1 cells as it was determined by multi-cycle growth kinetics. The result of Mean death time test indicates that the addition of ILTV genes did not increase the virulence of NDV strain LaSota vector. Finally, the genetic stability of rNDV's containing ILTV glycoprotein genes was evaluated by 10 serial passages in 9-day old SPF eggs and 10 serial passages in DF-1 cells followed by sequencing analysis for the evaluation of genomic integrity of the added genes.

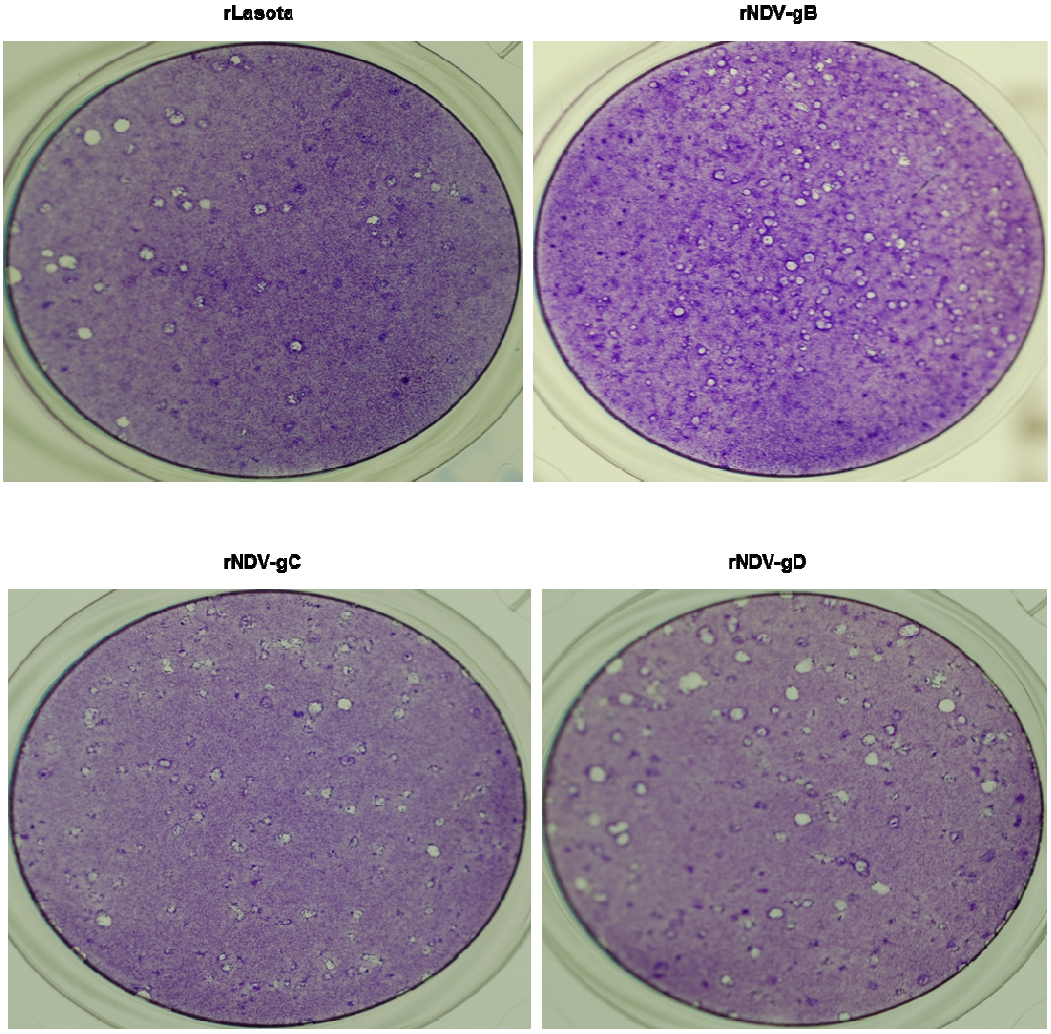

Figure 11. Plaque morphology of rNDV's expressing ILTV gB, gC and gD proteins.

4.3 Evaluation of immunogenicity and protective efficacy of recombinant NDVs expressing ILTV glycoproteins in chickens.

4.3.1 Immunogenicity and protective efficacy of rNDV's in chickens against highly-virulent NDV challenge

To evaluate protective efficacy, groups of chickens were inoculated by the oculonasal route with rNDV LaSota, or with the recombinant rNDV's expressing ILTV glycoproteins according to the protocol described in Table 4. The birds were challenged 21 days later by the oculonasal route with highly-virulent NDV strain Texas-GB. All of the chickens that had been immunized with rNDV LaSota or rNDV's expressing ILTV glycoproteins were completely protected from NDV challenge without any disease signs and with no evidence of challenge virus replication in the organs collected 3 days post challenge. In contrast, all of the chickens in the unvaccinated control group and CEO vaccinated group died within 3 days after challenge. Each of the recombinant NDV immunizations induced a substantial titer of NDV-specific HI serum antibodies when assayed on day 21 prior to challenge. However, the mean antibody titers induced by rNDV's expressing ILTV glycoproteins were approximately one or two log2 less than that of rNDV LaSota vaccine (Figure 12). This modest reduction in immune response to the vector might reflect increased attenuation of the rNDV associated with the insertion of the foreign gene, as indicated above by the MDT assay. The result of this study also suggests that expression of the ILTV glycoprotein do not interferes with protective immunity of NDV LaSota vaccine.

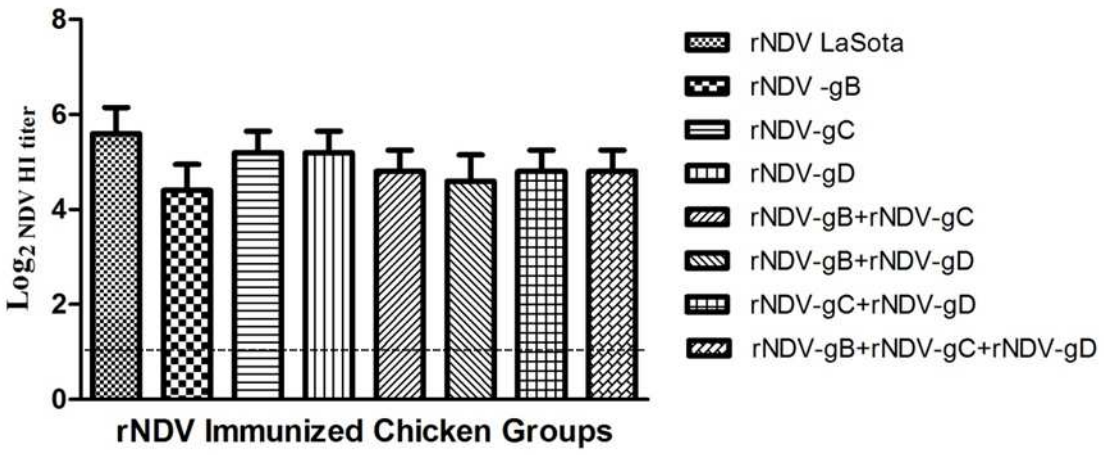

Figure 12. NDV-specific serum antibody response in chickens against rNDV's. NDV-specific serum antibody responses in chickens at 21 days following oculonasal immunization with the indicated rNDVs administered individually or in combination. All antibody titers are expressed as mean reciprocal log2 titer ± SEM (standard error of the mean), and the sample titers above the dotted line were considered positive. Statistical differences were calculated by one-way ANOVA with $P < 0.0001$.

4.3.2 Immunogenicity and protective efficacy of rNDV's in chickens against virulent USDA strain of ILTV challenge

4.3.2.1 Clinical signs score evaluation

Total clinical sign scores were recorded daily per group and are summarized in Figure 13a and Figure 13b. In figure 13a, total clinical scores of rNDV LaSota, rNDV-gB, rNDV-gC, rNDV-gD vaccinated groups were compared with clinical scores of Trachivax CEO vaccinated and unvaccinated groups. In figure 13b, the total clinical

scores of rNDV-gB+rNDV-gC, rNDV-gB+rNDV-gD, rNDV-gC+rNDV-gD and rNDV-gB+rNDV-gC+rNDV-gD vaccinated groups were compared with clinical scores of Trachivax CEO vaccinated and unvaccinated groups. Group of chickens in the control group and rNDV LaSota vaccinated group showed severe dyspnea with open mouth breathing, severe depression and severe conjunctivitis with closure of eyes between 2 to 9 d.p.i. with 3 mortalities in each group. Birds in the control group showed symptoms of the disease until 11th day post challenge. As expected, birds vaccinated with the Trachivax CEO vaccine did not show any signs of the disease except for very mild depression between 2nd and 3rd day post challenge. No mortalities were observed in Trachivax vaccinated birds. Birds vaccinated with rNDV-gB vaccine showed severe signs of dyspnea, depression and conjunctivitis between 2nd and 4th day post challenge with two mortalities recorded on 4th d.p.i. Disease signs were almost disappeared on day 6th post challenge for rNDV-gB vaccinated group. Birds vaccinated with rNDV-gC vaccine showed severe disease signs between 2nd and 6th d.p.i with a single mortality observed on 4th d.p.i. and disease signs were recorded until 8th day post infection. Birds vaccinated with rNDV-gD vaccine showed severe signs of disease between 3rd and 6th d.p.i. with two mortalities observed on 4th and 6th d.p.i. Disease signs were recorded until 8th day post infection for rNDV-gD vaccinated group. Birds vaccinated with multivalent vaccines containing rNDV's in various combinations (Figure b) showed severe signs of disease on 3rd and 4th d.p.i. with no evident clinical signs observed on day 6th post challenge. Group of chickens vaccinated with rNDV-gB+rNDV-gD vaccine showed one mortality and rNDV-gC+rNDV-gD vaccinated group showed 2 mortalities with no mortality observed for rNDV-gB+rNDV-gC and rNDV-gB+rNDV-gC+rNDV-

gD vaccinated groups. Statistical analysis of clinical signs scores between groups was precluded because scores were recorded as a group-level measurement, rather than measurement on individual birds per group.

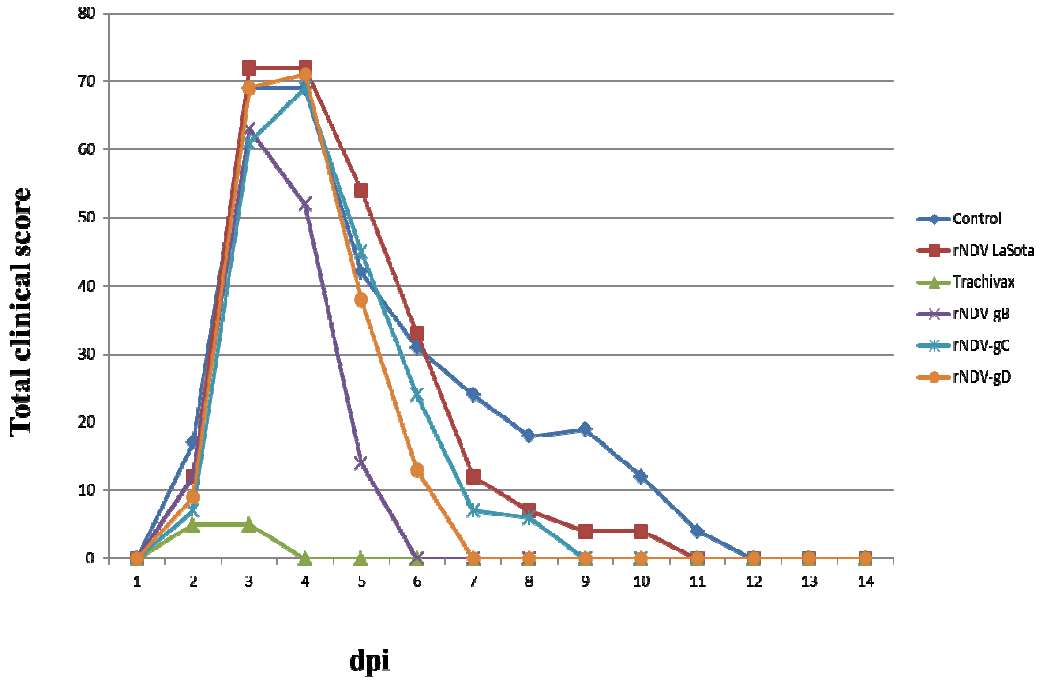

Figure 13a. Total clinical sign scores recorded daily for rNDV LaSota, rNDV-gB, rNDV-gC, and rNDV-gD vaccinated groups and compared with clinical scores recorded for control and Trachivax vaccinated groups.

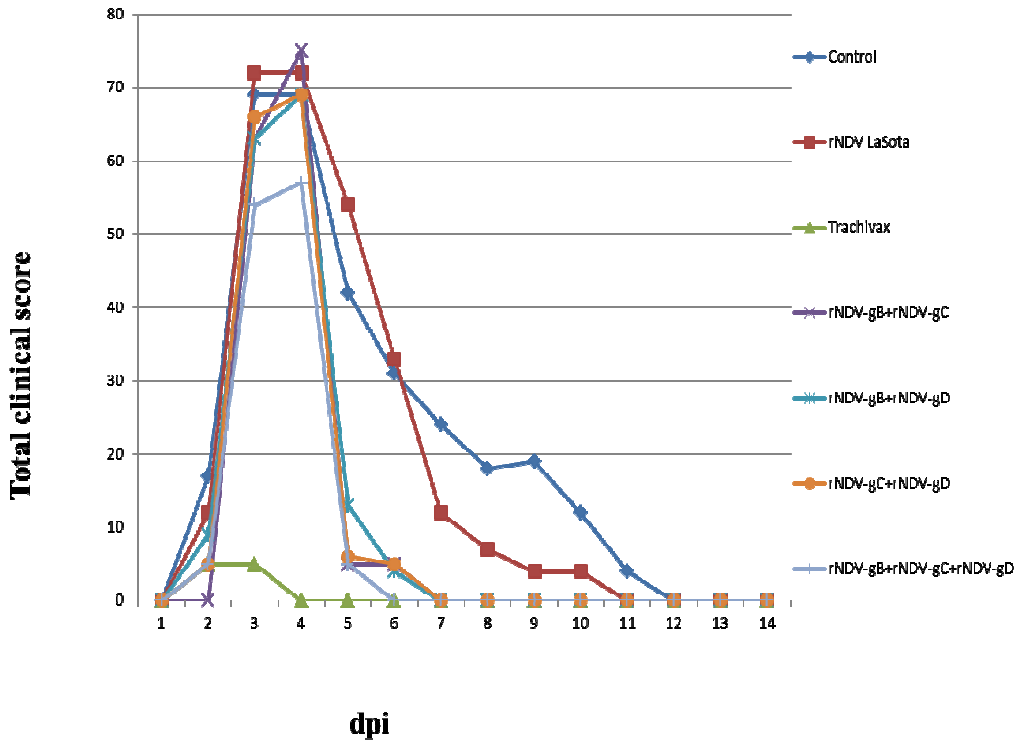

Figure 13b. Total clinical sign scores recorded daily for rNDV LaSota, rNDV-gB+rNDV-gC, rNDV-gB+rNDV-gD, and rNDV-gB+rNDV-gC+rNDV-gD vaccinated groups and compared with clinical scores recorded for control and Trachivax vaccinated groups.

4.3.2.2 ILTV loads in trachea post challenge

Viral load levels in the trachea were quantified at 4th day post challenge by TCID50 method and are presented in Table.6. There were no significant differences observed in viral load levels in the trachea between control group and rNDV's vaccinated group except for the group of chickens vaccinated with rNDV-gB+rNDV-gC vaccine which showed approximately 2 log10 lower ($P < 0.05$) viral load in trachea compared to control group. We were not able detect challenge virus in the tracheas of Trachivax vaccinated birds 4th day post challenge.

Group	Mean tracheal viral load at 4 day post challenge(log_{10}) for the indicated groups
Trachivax	0.0
Control	5.5 (±0.1155)
rNDV LaSota	5.5 (±0.05773)
rNDV-gB	5.2 (±0.5774)
rNDV-gC	4.9 (±0.6557)
rNDV-gD	5.5 (±0.0)
rNDV-gB+rNDV-gC	3.7 (±1.258)
rNDV-gB+rNDV-gD	4.7 (±1.443)
rNDV-gC+rNDV-gD	4.7 (±1.193)
rNDV-gB+rNDV-gC+rNDV-gD	3.2 (±1.258)

Table.6. Tracheal viral load in chickens on 4 day post challenge with USDA strain of ILTV.

4.3.2.3 Macroscopic changes in the trachea following ILTV challenge.

The tracheas collected from the experimental chickens following ILTV challenge were cut open longitudinally and observed for the macroscopic lesions. The tracheas collected from the chickens vaccinated with rNDV's and control birds showed severe hemorrhages throughout the trachea with bloody mucus discharge, whereas tracheas collected from the birds vaccinated with rNDV-gB+rNDV-gC+rNDV-gD vaccine showed mild lesions with only mucus discharge (Figure 14). As expected, tracheas collected from the Trachivax vaccinated birds did not show any lesions in the trachea which was correlated with the absence of challenge virus replication in trachea.

4.3.2.4 Amplification of TK gene from the DNA isolated from trigeminal ganglia.

DNA was isolated from the trigeminal ganglia of the birds collected 4 day post challenge using Qiagen's QIAamp DNA mini kit (250) according to the manufacturer protocols. The isolated DNA was used to amplify TK gene by nested PCR using the primers mentioned in the Table 5. We were not able to amplify the TK gene from the DNA isolated from trigeminal ganglia of any of the experimental birds (data not shown) indicating further standardization of the amplification protocols used in the technique.

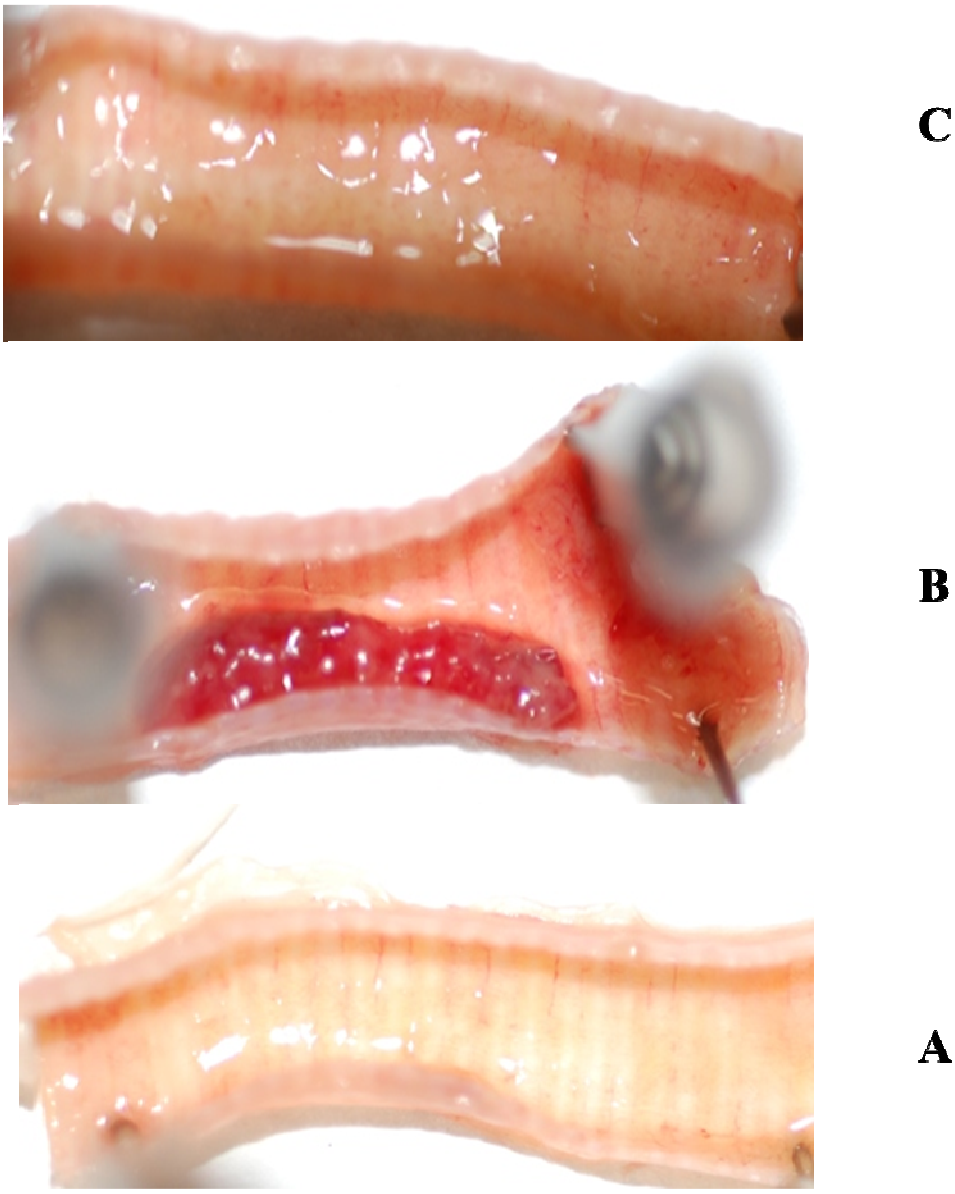

Figure 14. Macroscopic lesions in the trachea collected from birds 4th day post challenge. Panel A: Trachea from the CEO vaccine Trachivax vaccinated bird, Panel B: Trachea from the control bird (only control bird trachea is represented here since the lesions in the trachea from rNDV's vaccinated birds were similar to lesions seen in control bird trachea), Panel C: Trachea from the rNDV-gB+rNDV-gC+rNDV-gD vaccinated bird.

4.3.2.5 ILTV ELISA and virus neutralizing antibody titers.

The ILTV antibody response in pre-challenge and post-challenge sera was measured by ELISA and VN assay. The mean antibody titers in pre-challenge and post-challenge sera collected from vaccinated groups was determined by ELISA and is summarized in the Figure 15. Except for the Trachivax, rNDV-gC, rNDV-gB+rNDV-gC and rNDV-gB+rNDV-gC+rNDV-gD vaccinated groups; all the remaining serum samples were negative by ELISA for ILTV antibodies when the serum was assayed according to manufacturer protocols with the test serum dilution of 1:50. However, anti-ILTV antibodies were detected in the pre-challenge sera collected from rNDV's vaccinated birds when 1:10 dilution of test serum was used for ELISA. Post-challenge sera showed a mean ILTV antibody titer that ranged from 2000-7000 for rNDV's vaccinated groups and a mean titer of 13500 for Trachivax vaccinated group. ELISA titers for serum samples collected from the control group of chickens showed a mean titer of 1000 at 14 day post challenge. ILTV neutralizing antibodies were also determined in serum samples collected from the pre-challenge and post-challenge (Figure 16). Low virus neutralizing antibodies were detected in the pre-challenge sera with mean titers ranging from 2-4 (log2) for all the vaccinated groups. Post- challenge serum samples showed a mean virus neutralizing antibody titers ranging from 4 to 8 (log2) for rNDV's vaccinated birds and surprisingly, the neutralizing antibody titers in the post challenge sera from Trachivax vaccinated birds were of low titer in the range of 1-2 (log2) and such titers were not different from the titers obtained for the control group.

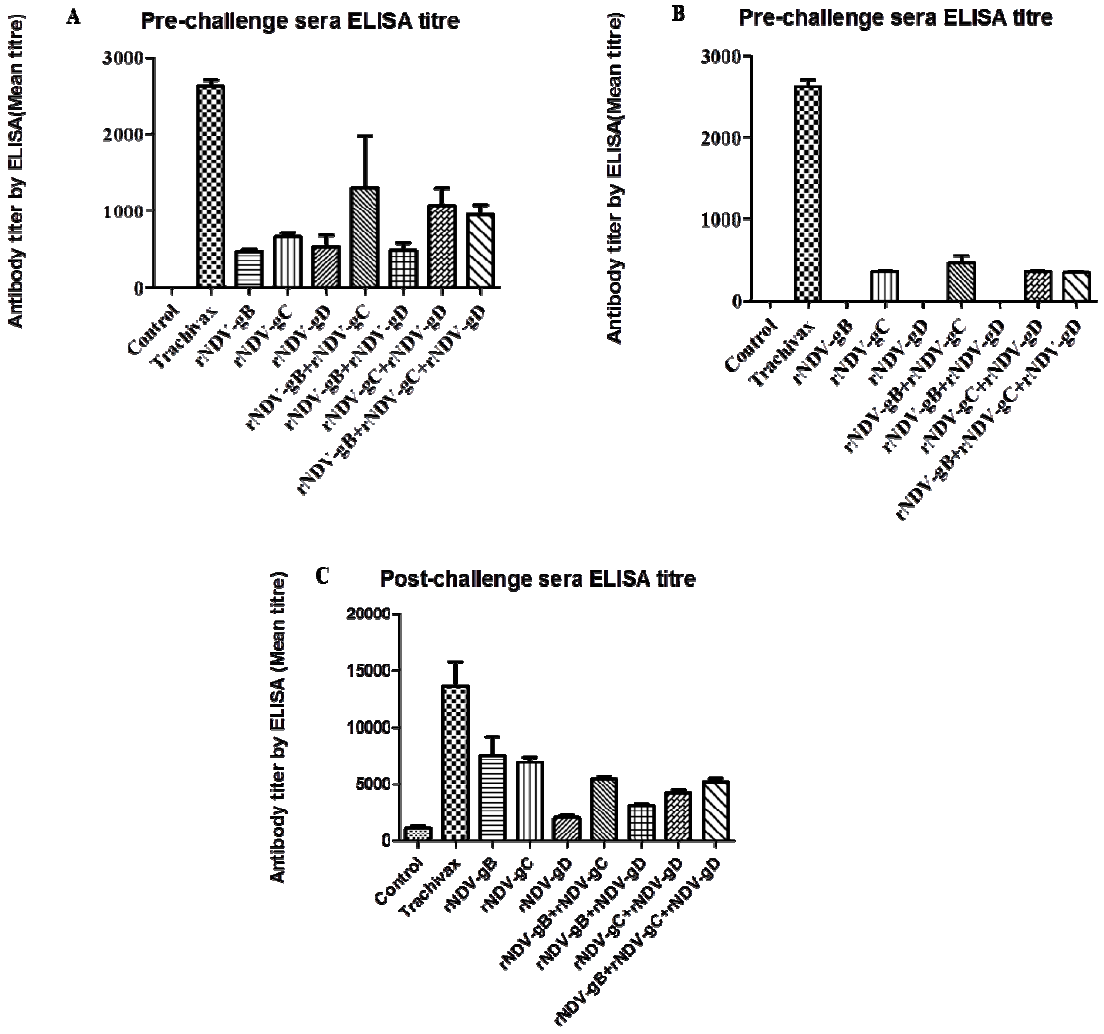

Figure 15. ILTV antibody titers determined by ELISA on serum samples collected pre-challenge and post-challenge. A: Pre-challenge ILTV antibody titer when 1:10 dilution of test serum used, B: Pre- challenge ILTV antibody titer when 1:50 dilution of test serum used, C: Post-challenge ILTV antibody titer.

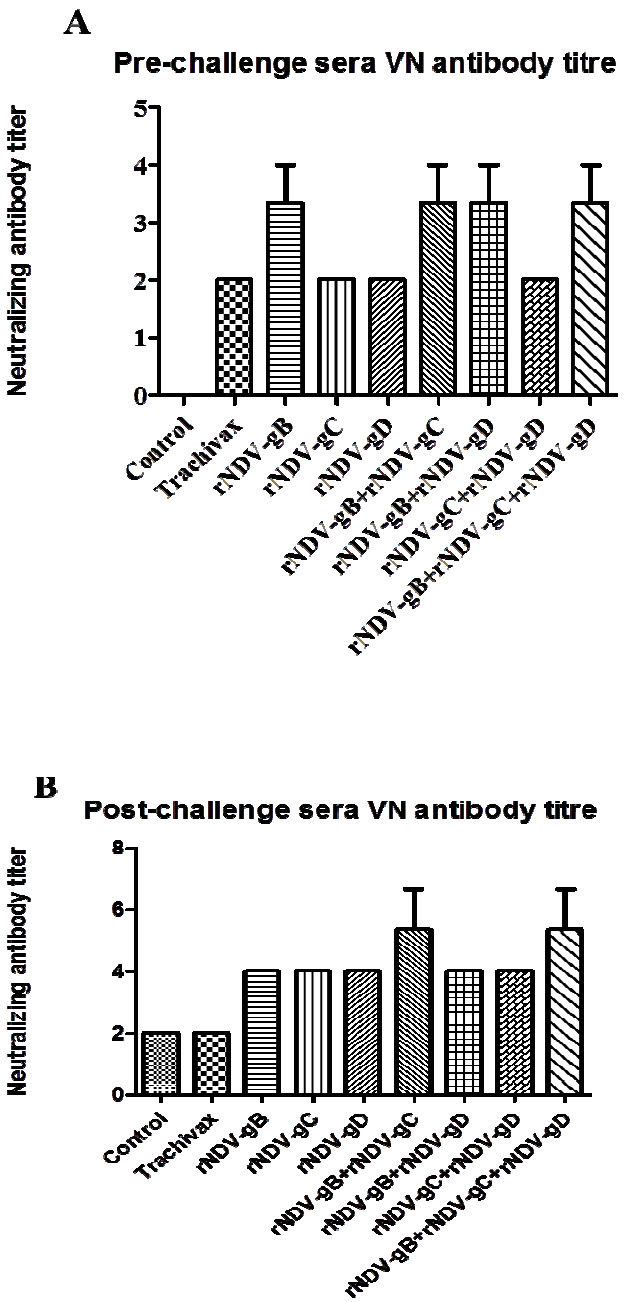

Figure 16. . ILTV antibody titers determined by VN assay on serum samples collected pre-challenge and post-challenge. A: Pre-challenge ILTV antibody titer and, B: Post-challenge ILTV antibody titer.

4.3.3 Conclusions.

Currently gB is the only ILTV glycoprotein whose role has been evaluated in protective immunity [22]. However, the role of other ILTV glycoproteins in protection and immunity has not been studied. Therefore, a virulent challenge study was carried out in chickens to evaluate the individual contributions of ILTV gB, gC, and gD surface proteins to the induction of neutralizing antibodies and protective immunity. It is possible that more than one ILTV glycoprotein is essential for induction of complete protective immunity. Therefore, we have also evaluated these three glycoproteins in all possible combinations via multivalent vaccine strategy consisting of a mixture of rNDVs each carrying different genes (gB, gC and gD) of ILTV. A single oculonasal inoculation of chickens with rNDV's expressing ILTV glycoproteins elicited detectable level of systemic antibodies specific to expressed ILTV glycoproteins with the response to rNDV-gC being higher than those to rNDV-gB or rNDV-gD. Following challenge with virulent USDA challenge strain of ILTV, chickens immunized with the recombinant NDV's displayed partial protection with reduced clinical signs and shorter duration of disease compared to control group. The rNDV vectored vaccines fails to prevent the challenge virus replication in trachea following challenge with USDA strain of ILTV. However, chickens immunized with the multivalent vaccine consisting of rNDV-gB, rNDV-gC and rNDV-gD viruses exhibited reduced challenge virus replication in trachea compared to control birds. Following challenge, the titers of serum antibodies specific to ILTV glycoproteins were higher in the animals immunized with the rNDV vaccines compared to the rNDV parent virus, indicating that the vaccines primed for secondary responses. Our data suggest that NDV vectored ILTV vaccines are useful against ILTV

infection, but might require augmentation by a second dose or require modification of ILTV glycoproteins which allow them to incorporate into the mature rNDV virions for better induction of humoral and CMI response. Further, the results of the present study strongly suggests that the multivalent vaccines consisting of rNDV's expressing ILTV gB, gC and gD is very effective in clearing the infection in an accelerated time frame and also reduced the challenge virus replication in trachea and mitigated the clinical signs of the disease significantly.

Chapter 5: Discussion

ILT is a highly contagious disease of chickens worldwide. The disease causes significant economic losses due to morbidity, mortality, decreased egg production, weight loss, and predisposition to other avian pathogens [1, 46]. Traditionally, ILTV is controlled by the use of modified-live vaccines. However, current modified-live ILTV vaccines have a number of disadvantages including insufficient attenuation, production of latently infected carriers, and increased virulence as a result of bird-to-bird passage (2, 3, 4). Therefore, there is a need to develop alternative vaccine strategies for ILT which can be used in both layers and broilers. Among the possible strategies, one of the most promising is the use of live viral vectored vaccines. The major advantage of a live viral vectored vaccine is that they do not require the use of the whole infectious pathogen but can have the efficacy of live-attenuated vaccine. Several studies conducted to date suggest that a virus-vectored ILTV vaccine will be most effective for prevention and control of ILT. A vectored-vaccine will be safe and not lead to reversion to virulence or establishment of latency (22, 58). Several viruses such as fowlpox, adenovirus, adeno-associated virus, NDV and HVT are available for use as vaccine vectors. But each viral vector has its own advantages and disadvantages for use as a vaccine vector for ILTV. NDV has a several feature that make it a promising viral vector for ILTV vaccine. Avirulent NDV strains have been widely used as vaccines for more than 50 years with a proven record of safety and efficacy. NDV is a respiratory pathogen and efficiently induces mucosal immunity at the respiratory tract, which is also the site of replication for ILTV. NDV induces strong systemic IgG antibody and CMI responses [15]. NDV is a relatively small virus with six genes; therefore, chances are better for a lack of significant

immune competition between engineered ILTV antigen and NDV-specific antigens. A NDV-vectored ILTV vaccine can be used as early as one day of age; therefore, it can be used in broiler chickens. NDV-vectored vaccines can be administered by spray or in drinking water, which will allow vaccination of a large number of birds in a short time. Chickens in the United States are routinely vaccinated with live-attenuated NDV vaccine. Therefore, NDV-vectored ILTV vaccine will be a bivalent vaccine and will not require introduction of a new vaccine. Another advantage of the NDV-vectored ILTV vaccine is that vaccinated chickens can be distinguished from naturally ILTV infected ones by serological tests. Therefore, development of an NDV-vectored ILTV vaccine will be highly beneficial to the poultry industry in the United States. In this study, for the first time, we have evaluated the potential of NDV as a viral vector for ILTV vaccine.

ILTV infection predominantly affects the mucosa and submucosa of the upper respiratory tract of chickens and leads to the induction of both mucosal and systemic immune response (49, 50). ILTV has eleven envelope glycoproteins and gB has been shown to be a major protective immunogen [22], but the roles of gC and gD in immunity and protection have not been determined. In the present study, we have investigated the individual contributions of three important envelope glycoproteins of ILTV (gB, gC, and gD) in protection and immunity.

A single oculonasal inoculation of chickens with rNDV's expressing ILTV glycoproteins elicited detectable level of systemic antibodies specific to expressed ILTV glycoproteins with the response to rNDV-gC being higher than those to rNDV-gB or rNDV-gD. The immune response produced by a single immunization with these rNDV's was not sufficient to prevent the appearance of clinical signs and the challenge virus

replication in trachea, but reduced the mortality, severity and the duration of disease compared to the control group. The increase of ILTV specific antibody titers in vaccinated chickens following challenge suggested that vaccine primed for secondary response. Detection of antibodies against expressed ILTV glycoproteins was not interpreted as a measure of protection, but rather as a measure of antigenicity of ILTV glycoproteins expressed by the vector. However, the presence of low titers of ILTV specific antibodies in the pre-challenge sera coincided with the presence of clinical signs and high level of challenge virus in the trachea. On the other hand, the presence of higher titers of ILTV antibodies in the pre-challenge sera collected from rNDV-gB+rNDV-gC and rNDV-gB+rNDV-gC+rNDV-gD immunized birds coincided with the reduced challenge virus replication in trachea and reduced mortality, severity and duration of the disease. Although direct evidence of major effector mechanisms involved in ILTV immune response is lacking, it has been documented that humoral antibody response by itself does not provide the immunity necessary for protection against ILTV infections (50, 51, 87, 88). The minor role that antibodies play in immunity against ILTV infections is further confirmed by the absence of measurable levels of neutralizing antibodies in the sera collected from CEO vaccinated birds. Therefore, to thoroughly assess the immunogenicity and protective efficacy of rNDV vectored ILTV vaccines, the mucosal and cell-mediated immunity would need to be evaluated.

In the present study, the failure to provide complete protection against ILTV by the rNDV's expressing ILTV glycoproteins likely was due to insufficient induction of systemic immune response. Previous studies have also reported varying degree of protection using Fowl pox and Herpes virus of Turkey viral vectors (22, 58, 59, 60).

There could be many reasons for the partial protection conferred by NDV vectored ILTV vaccines in this study. First, it is possible that repetitive doses of the rNDV's expressing ILTV proteins are necessary to augment the systemic immune response for complete protection. Second, in the present study, only ILTV-gC is found to be incorporated into the mature rNDV virion which leads to the better antigen presentation and hence induction of humoral immune response in chickens immunized with rNDV-gC vaccine compared to rNDV-gB or rNDV-gD vaccine. Therefore, ILTV gB and gD glycoproteins require certain modifications which allow them to incorporate into the mature rNDV virions for better induction of humoral response and therefore better CMI response. Third, in this experiment chickens were challenged with a high dose of virulent USDA challenge strain of ILTV. Such a high dose of infections does not occur under natural conditions. Hence overwhelming the immune response by challenge virus exists.

In future directions, the following strategies can be employed to further improve the rNDV based ILTV vaccines. First of all, in the current study only ILTV gC was found to be incorporated into the mature rNDV virion and rNDV expressing ILTV gC can readily activate the antigen presenting cells and B-cells for the better induction of humoral response which is coincided with the better antibody response to rNDV-gC compared to rNDV-gB or rNDV-gD. Also, induced antibodies can have an opsonizing ability to further improve the cell mediated immune responses. Therefore, ILTV gB and gD proteins requires certain modification that allow them to incorporate into the mature rNDV virion. To achieve this, one of the most promising strategy is to fuse the ectodomain of ILTV gB and ILTV gD proteins to transmembrane and cytoplasmic tail of NDV 'F' protein. This will allow them to incorporate into the mature rNDV virion. This

strategy is very straightforward and has been demonstrated to be very useful in enhancing the incorporation of foreign proteins into envelope of mature rNDV's (13). Further, Immune response to mature rNDV envelope incorporated ILTV proteins can be improved by use of repetitive doses of vaccines and also by use of killed vaccines to minimize the immune competition between ILTV proteins and NDV envelope proteins. Secondly, it might necessitate the inclusion of additional ILTV antigens for complete protection. It is known that ILTV glycoproteins have homologous glycoproteins in other herpesviruses and It has been shown that in herpes simplex virus (HSV) gB, gC, gD, gE and gI induced highest neutralizing titers and protective immunity [6]. Therefore, the incomplete protection generated by vaccination with NDV vectors expressing only gB, gC and gD might be overcome by simultaneously administering NDV vectors expressing the gB, gC, gD, gE and gI proteins.

In summary, for the first time we have evaluated the potential of rNDV as a viral vector for ILTV vaccine. Our results showed that chickens immunized with recombinant viruses elicited an immune response against ILTV proteins and provided partial protection against virulent ILTV challenge. These results suggested that ILTV envelope glycoproteins gB, gC and gD could be a useful components of live viral vectored vaccines for ILTV infection. Furthermore, this strategy may be useful for the development of live viral vectored vaccines against ILT for which currently safe and efficacious vaccines are not available.

Bibliography

1. Bagust TJ, Jones RC, Guy JS: Avian infectious laryngotracheitis. Rev Sci Tech 2000, 19(2):483-492.

2. Guy JS, Barnes HJ, Smith L: Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis 1991, 35(2):348-355.

3. Hughes CS, Williams RA, Gaskell RM, Jordan FT, Bradbury JM, Bennett M, Jones RC: Latency and reactivation of infectious laryngotracheitis vaccine virus. Arch Virol 1991, 121(1-4):213-218.

4. Clarke JK, Robertson GM, Purcell DA: Spray vaccination of chickens using infectious laryngotracheitis virus. Aust Vet J 1980, 56(9):424-428.

5. Chang PC, Chen KT, Shien JH, Shieh HK: Expression of infectious laryngotracheitis virus glycoproteins in Escherichia coli and their application in enzyme-linked immunosorbent assay. Avian Dis 2002, 46(3):570-580.

6. Ghiasi H, Kaiwar R, Nesburn AB, Slanina S, Wechsler SL: Expression of seven herpes simplex virus type 1 glycoproteins (gB, gC, gD, gE, gG, gH, and gI): comparative protection against lethal challenge in mice. J Virol 1994, 68(4):2118-2126.

7. Mettenleiter TC (ed.): Molecular Biology of Animal Herpesviruses. Norfolk, UK: Caister Academic Press; 2008.

8. Wang XK, X. Y. Tang, G. X. Xin, and Q. X. Liu. : Development of neutralization test for avian infectious laryngotracheitis. Chin J Anim Infect Dis 1982, 4:13-17.

9. York JJ, Fahey KJ: Vaccination with affinity-purified glycoproteins protects chickens against infectious laryngotracheitis herpesvirus. Avian Pathol 1991, 20(4):693-704.

10. Huang Z, Krishnamurthy S, Panda A, Samal SK: High-level expression of a foreign gene from the most 3'-proximal locus of a recombinant Newcastle disease virus. J Gen Virol 2001, 82(Pt 7):1729-1736.

11. Khattar SK, Collins PL, Samal SK: Immunization of cattle with recombinant Newcastle disease virus expressing bovine herpesvirus-1 (BHV-1) glycoprotein D induces mucosal and serum antibody responses and provides partial protection against BHV-1. Vaccine, 28(18):3159-3170.

12. Nayak B, Kumar S, DiNapoli JM, Paldurai A, Perez DR, Collins PL, Samal SK: Contributions of the avian influenza virus HA, NA, and M2 surface proteins to the induction of neutralizing antibodies and protective immunity. J Virol, 84(5):2408-2420.

13. Nayak B, Rout SN, Kumar S, Khalil MS, Fouda MM, Ahmed LE, Earhart KC, Perez DR, Collins PL, Samal SK: Immunization of chickens with Newcastle disease virus expressing H5 hemagglutinin protects against highly pathogenic H5N1 avian influenza viruses. PLoS One 2009, 4(8):e6509.

14. Huang Z, Elankumaran S, Yunus AS, Samal SK: A recombinant Newcastle disease virus (NDV) expressing VP2 protein of infectious bursal disease virus (IBDV) protects against NDV and IBDV. J Virol 2004, 78(18):10054-10063.

15. DiNapoli JM, Yang L, Suguitan A, Jr., Elankumaran S, Dorward DW, Murphy BR, Samal SK, Collins PL, Bukreyev A: Immunization of primates with a Newcastle disease virus-vectored vaccine via the respiratory tract induces a high titer of serum neutralizing antibodies against highly pathogenic avian influenza virus. J Virol 2007, 81(21):11560-11568.

16. DiNapoli JM, Kotelkin A, Yang L, Elankumaran S, Murphy BR, Samal SK, Collins PL, Bukreyev A: Newcastle disease virus, a host range-restricted virus, as a vaccine vector for intranasal immunization against emerging pathogens. Proc Natl Acad Sci U S A 2007, 104(23):9788-9793.

17. DiNapoli JM, Nayak B, Yang L, Finneyfrock BW, Cook A, Andersen H, Torres-Velez F, Murphy BR, Samal SK, Collins PL et al: Newcastle disease virus-vectored vaccines expressing the hemagglutinin or neuraminidase protein of H5N1 highly pathogenic avian influenza virus protect against virus challenge in monkeys. J Virol, 84(3):1489-1503.

18. DiNapoli JM, Ward JM, Cheng L, Yang L, Elankumaran S, Murphy BR, Samal SK, Collins PL, Bukreyev A: Delivery to the lower respiratory tract is required for effective immunization with Newcastle disease virus-vectored vaccines intended for humans. Vaccine 2009, 27(10):1530-1539.

19. Rajcani J, Vojvodova A: The role of herpes simplex virus glycoproteins in the virus replication cycle. Acta Virol 1998, 42(2):103-118.

20. Bender FC, Whitbeck JC, Lou H, Cohen GH, Eisenberg RJ: Herpes simplex virus glycoprotein B binds to cell surfaces independently of heparan sulfate and blocks virus entry. J Virol 2005,79(18):11588-11597.

21. Kwon H, Bai Q, Baek HJ, Felmet K, Burton EA, Goins WF, Cohen JB, Glorioso JC: Soluble V domain of Nectin-1/HveC enables entry of herpes simplex virus type 1 (HSV-1) into HSV-resistant cells by binding to viral glycoprotein D. J Virol 2006, 80(1):138-148.

22. Tong GZ, Zhang SJ, Wang L, Qiu HJ, Wang YF, Wang M: Protection of chickens from infectious laryngotracheitis with a recombinant fowlpox virus expressing glycoprotein B of infectious laryngotracheitis virus. Avian Pathol 2001, 30(2):143-148.

23. Gogev S, Georgin JP, Schynts F, Vanderplasschen A, Thiry E: Bovine herpesvirus 1 glycoprotein D expression in bovine upper respiratory tract mediated by a human adenovirus type 5. Vet Res 2004, 35(6):715-721.

24. Gogev S, Vanderheijden N, Lemaire M, Schynts F, D'Offay J, Deprez I, Adam M, Eloit M, Thiry E: Induction of protective immunity to bovine herpesvirus type 1 in cattle by intranasal administration of replication-defective human adenovirus type 5 expressing glycoprotein gC or gD. Vaccine 2002, 20(9-10):1451-1465.

25. Fuchs W, Veits J, Helferich D, Granzow H, Teifke JP, Mettenleiter TC: Molecular biology of avian infectious laryngotracheitis virus. Vet Res 2007, 38(2):261-279.

26. Thureen DR, Keeler CL, Jr.: Psittacid herpesvirus 1 and infectious laryngotracheitis virus: Comparative genome sequence analysis of two avian alphaherpesviruses. J Virol 2006, 80(16):7863-7872.

27. Fuchs W, Granzow H, Klupp BG, Karger A, Michael K, Maresch C, Klopfleisch R, Mettenleiter TC: Relevance of the interaction between alphaherpesvirus UL3.5 and UL48 proteins for virion maturation and neuroinvasion. J Virol 2007, 81(17):9307-9318.

28. Rodriguez-Avila A, Oldoni I, Riblet S, Garcia M: Evaluation of the protection elicited by direct and indirect exposure to live attenuated infectious

laryngotracheitis virus vaccines against a recent challenge strain from the United States. Avian Pathol 2008, 37(3):287-292.

29. Oldoni I, Rodriguez-Avila A, Riblet S, Garcia M: Characterization of infectious laryngotracheitis virus (ILTV) isolates from commercial poultry by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Avian Dis 2008, 52(1):59-63.

30. Fischer L, Barzu S, Andreoni C, Buisson N, Brun A, Audonnet JC: DNA vaccination of neonate piglets in the face of maternal immunity induces humoral memory and protection against a virulent pseudorabies virus challenge. Vaccine 2003, 21(15):1732-1741.

31. Hong W, Xiao S, Zhou R, Fang L, He Q, Wu B, Zhou F, Chen H: Protection induced by intramuscular immunization with DNA vaccines of pseudorabies in mice, rabbits and piglets. Vaccine 2002, 20(7-8):1205-1214.

32. Lukacs N, Thiel HJ, Mettenleiter TC, Rziha HJ: Demonstration of three major species of pseudorabies virus glycoproteins and identification of a disulfide-linked glycoprotein complex. J Virol 1985, 53(1):166-173.

33. Hampl H, Ben-Porat T, Ehrlicher L, Habermehl KO, Kaplan AS: Characterization of the envelope proteins of pseudorabies virus. J Virol 1984, 52(2):583-590.

34. Zuckermann FA, Zsak L, Mettenleiter TC, Ben-Porat T: Pseudorabies virus glycoprotein gIII is a major target antigen for murine and swine virus-specific cytotoxic T lymphocytes. J Virol 1990, 64(2):802-812.

35. Ober BT, Summerfield A, Mattlinger C, Wiesmuller KH, Jung G, Pfaff E, Saalmuller A, Rziha HJ: Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+CD8+ memory T-helper cells in swine. J Virol 1998, 72(6):4866-4873.

36. Babiuk LA, L'Italien J, van Drunen Littel-van den Hurk S, Zamb T, Lawman JP, Hughes G, Gifford GA: Protection of cattle from bovine herpesvirus type I (BHV-1) infection by immunization with individual viral glycoproteins. Virology 1987, 159(1):57-66.

37. Chase CC, Carter-Allen K, Letchworth GJ, 3rd: The effect of bovine herpesvirus type 1 glycoproteins gI and gIII on herpesvirus infections. J Gen Virol 1989, 70 (Pt 6):1561-1569.

38. van Drunen Littel-van den Hurk S, Gifford GA, Babiuk LA: Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. Vaccine 1990, 8(4):358-368.

39. Gao Y, Leary TP, Eskra L, Splitter GA: Truncated bovine herpesvirus-1 glycoprotein I (gpI) initiates a protective local immune response in its natural host. Vaccine 1994, 12(2):145-152.

40. Zhu X, Letchworth GJ, 3rd: Mucosal and systemic immunity to bovine herpesvirus-1 glycoprotein D confer resistance to viral replication and latency in cattle. Vaccine 1996, 14(1):61-69.

41. Russell RG, Turner AJ: Characterization of infectious laryngotracheitis viruses, antigenic comparison by kinetics of neutralization and immunization studies. Can J Comp Med 1983, 47(2):163-171.

42. Izuchi T, Hasegawa A: Pathogenicity of infectious laryngotracheitis virus as measured by chicken embryo inoculation. Avian Dis 1982, 26(1):18-25.

43. Jones RC: Viral respiratory diseases (ILT, aMPV infections, IB): are they ever under control? Br Poult Sci, 51(1):1-11.

44. Portz C, Beltrao N, Furian TQ, Junior AB, Macagnan M, Griebeler J, Lima Rosa CA, Colodel EM, Driemeier D, Back A et al: Natural infection of turkeys by infectious laryngotracheitis virus. Vet Microbiol 2008, 131(1-2):57-64.

45. Winterfield RW, So IG: Susceptibility of turkeys to infectious laryngotracheitis. Avian Dis 1968, 12(1):191-202.

46. Bagust TJ: Laryngotracheitis (Gallid-1) herpesvirus infection in the chicken. 4. Latency establishment by wild and vaccine strains of ILT virus. Avian Pathol 1986, 15(3):581-595.

47. Williams RA, Bennett M, Bradbury JM, Gaskell RM, Jones RC, Jordan FT: Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. J Gen Virol 1992, 73 (Pt 9):2415-2420.

48. Kaleta EF, Redmann T, Heffels-Redmann U, Frese K: [Detection of attenuated virus latency in infectious laryngotracheitis in the trigeminal ganglion of the chicken]. Dtsch Tierarztl Wochenschr 1986, 93(1):40-42.

49. Honda T, Taneno A, Sakai E, Yamada S, Takahashi E: Immune response and in vivo distribution of the virus in chickens inoculated with the cell-associated vaccine of attenuated infectious laryngotracheitis (ILT) virus. J Vet Med Sci 1994, 56(4):691-695.

50. Fahey KJ, York JJ: The role of mucosal antibody in immunity to infectious laryngotracheitis virus in chickens. J Gen Virol 1990, 71 (Pt 10):2401-2405.

51. York JJ, Fahey KJ: Humoral and cell-mediated immune responses to the glycoproteins of infectious laryngotracheitis herpesvirus. Arch Virol 1990, 115(3-4):289-297.

52. Kotiw M, Wilks CR, Sheppard M, May JT: Comparison of methods for the isolation and purification of infectious laryngotracheitis virus DNA. Vet Microbiol 1984, 9(4):415-419.

53. Guy JS, Barnes HJ, Morgan LM: Virulence of infectious laryngotracheitis viruses: comparison of modified-live vaccine viruses and North Carolina field isolates. Avian Dis 1990, 34(1):106-113.

54. Oldoni I, Rodriguez-Avila A, Riblet SM, Zavala G, Garcia M: Pathogenicity and growth characteristics of selected infectious laryngotracheitis virus strains from the United States. Avian Pathol 2009, 38(1):47-53.

55. Han MG, Kweon CH, Mo IP, Kim SJ: Pathogenicity and vaccine efficacy of a thymidine kinase gene deleted infectious laryngotracheitis virus expressing the green fluorescent protein gene. Arch Virol 2002, 147(5):1017-1031.

56. Henderson LM, Levings RL, Davis AJ, Sturtz DR: Recombination of pseudorabies virus vaccine strains in swine. Am J Vet Res 1991, 52(6):820-825.

57. Henderson LM, Katz JB, Erickson GA, Mayfield JE: In vivo and in vitro genetic recombination between conventional and gene-deleted vaccine strains of pseudorabies virus. Am J Vet Res 1990, 51(10):1656-1662.

58. Sun HL, Wang YF, Tong GZ, Zhang PJ, Miao DY, Zhi HD, Wang M: Protection of chickens from Newcastle disease and infectious laryngotracheitis with a recombinant fowlpox virus co- expressing the F, HN genes of Newcastle disease virus and gB gene of infectious laryngotracheitis virus. Avian Dis 2008, 52(1):111-117.

59. Saif Y, Rosenberger J, Cloud M, Wild J, McMillen, Schwartz R: Efficacy and safety of a recombinant herpesvirus of turkeys containing genes from infectious laryngotracheitis virus. In: Proc Am Vet Med Assoc: 1994; Minneapolis, MN; 1994: 154.

60. Davidson I, Nagar S, Ribshtein I, Shkoda I, Perk S, Garcia M: Detection of wild- and vaccine-type avian infectious laryngotracheitis virus in clinical samples and feather shafts of commercial chickens. Avian Dis 2009, 53(4):618-623.

61. Alexander D: Newcastle disease. In: A laboratory manual for the isolation and identification of avian pathogens. Edited by H.G Purchase LHA, C.H.Domermuth and J.E Pearson, 3rd edn: American Association for Avian Pathologists, Inc., Kennett Square, Pa; 1989.

62. Meulemans G: Control by vaccination. In: Newcastle Disease. Edited by DJ A. Boston: Kluwer Academic Publications; 1988: 318–332.

63. Fauquet CM MM, Maniloff J, Desselberger U, Ball LA: Virus taxonomy. VIIIth report of the international committee on taxonomy of viruses. Elsevier Academic Press, San Diego 2005.

64. Krishnamurthy S, Huang Z, Samal SK: Recovery of a virulent strain of newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. Virology 2000, 278(1):168-182.

65. Steward M, Vipond IB, Millar NS, Emmerson PT: RNA editing in Newcastle disease virus. The Journal of general virology 1993, 74 (Pt 12):2539-2547.

66. Peeples M: Newcastle disease virus replication. In: Newcastle disease. Edited by Alexander D: Kluwer Academic publishers, Dordrecht, The Netherlands; 1988: 45-78.

67. Steward M, Samson AC, Errington W, Emmerson PT: The Newcastle disease virus V protein binds zinc. Archives of virology 1995, 140(7):1321-1328.

68. Lamb RA., Parks G: Paramyxoviridae: the viruses and their replication., (5th ed.) edn: Lippincott Williams and Wilkins, Philadelphia 2007.

69. Fuerst TR, Niles EG, Studier FW, Moss B: Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proceedings of the National Academy of Sciences of the United States of America 1986, 83(21):8122-8126.

70. Wyatt LS, Moss B, Rozenblatt S: Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology 1995, 210(1):202- 205.

71. Buchholz UJ, Finke S, Conzelmann KK: Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. Journal of virology 1999, 73(1):251-259.

72. Schnell MJ, Mebatsion T, Conzelmann KK: Infectious rabies viruses from cloned cDNA. The EMBO journal 1994, 13(18):4195-4203.

73. Baron MD, Barrett T: Rescue of rinderpest virus from cloned cDNA. Journal of virology 1997, 71(2):1265-1271.

74. Collins PL, Hill MG, Camargo E, Grosfeld H, Chanock RM, Murphy BR: Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proceedings of the National Academy of Sciences of the United States of America 1995, 92(25):11563-11567

75. Garcin D, Pelet T, Calain P, Roux L, Curran J, Kolakofsky D: A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus. The EMBO journal 1995, 14(24):6087-6094.

76. He B, Paterson RG, Ward CD, Lamb RA: Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. Virology 1997, 237(2):249-260.

77. Radecke F, Spielhofer P, Schneider H, Kaelin K, Huber M, Dotsch C, Christiansen G, Billeter MA: Rescue of measles viruses from cloned DNA. The EMBO journal 1995, 14(23):5773-5784.

78. Paldurai A, Kumar S, Kim S, Samal S: Role of genome lengths in Newcastle disease virus pathogenesis and virulence. In: The American Society for Virology. Montana; 2010.

79. Kolakofsky D, Roux L, Garcin D, Ruigrok RW: Paramyxovirus mRNA editing, the "rule of six" and error catastrophe: a hypothesis. The Journal of general virology 2005, 86(Pt 7):1869-1877.

80. Bukreyev A, Lamirande EW, Buchholz UJ, Vogel LN, Elkins WR, St Claire M, Murphy BR, Subbarao K, Collins PL: Mucosal immunisation of African green monkeys (Cercopithecus aethiops) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. Lancet 2004, 363(9427):2122-2127.

81. Skiadopoulos MH, Surman SR, Durbin AP, Collins PL, Murphy BR: Long nucleotide insertions between the HN and L protein coding regions of human parainfluenza virus type 3 yield viruses with temperature-sensitive and attenuation phenotypes. Virology 2000, 272(1):225- 234.

82. Summers MD, and Smith, G. E.: A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. . Texas Agricultural Experimental Station Bulletin 1987, 1555.

83. Alexander DJ (ed.): Avian paramyxoviruses 2-9: Ames: Iowa State University Press; 2003.

84. Oldoni I, Garcia M: Characterization of infectious laryngotracheitis virus isolates from the US by polymerase chain reaction and restriction fragment length polymorphism of multiple genome regions. Avian Pathol 2007, 36(2):167-176.

85. Swayne DE, Glisson JR, Jackwood MW, Pearson JE, WM R (eds.): A laboratory manual for the isolation and identification of avian pathogens., 4th edn. Iowa: Kindall/Hunt publishing Company; 1998.

86. Hierholzer JC, Killington RA (1996) Virus isolation and quantitation. In: Mahy BW, Kangro HI, editors. London, United Kingdom: Virology methods manual Academic Press. pp. 25–46.

87. Fahey, K.J., Bagust, T.J. & York, J.J (1983). Laryngotracheitis herpesvirus infection in the chicken: the role of humoral antibody in immunity to a graded challenge infection. Avian Pathology, 12, 505-514.

88. Honda, T., Okamura, H., Taneno, A., Yamada, S., & Takahashi, E. (1994). The role of cell-mediated immunity in chickens inoculated with the cell-associated vaccine of attenuated infectious laryngotracheitis virus. Journal of Veterinary Medical Science, 56, 1051-1055.